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(57) Abstract

The present invention provides compositions with high affinity for a target transcription factor, that can be introduced into cells as decoy *cis*-elements to bind the factor and alter gene expression. Specifically, the present invention provides nucleic acid molecules that complete with cAMP response element (CRE) enhancers for binding to transcription factors.

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TRANSCRIPTION FACTOR DECOY AND TUMOR GROWTH INHIBITOR

FIELD OF THE INVENTION

The present invention provides methods and compositions relating to oligonucleotides, with high affinity for a target transcription factor, that can be introduced into cells as decoy *cis*-elements to bind the factor and alter gene expression.

BACKGROUND OF THE INVENTION

A major focus of cellular and molecular research has concentrated on developing means to regulate gene expression (*i.e.*, gene transcription and translation) in an effort to treat and cure a variety of disease and conditions. It is hoped that the up- or down-regulation of specific genes will alter or circumvent the molecular mechanisms underlying these diseases and conditions. The importance of such research has dramatically increased as the Human Genome Project continues to identify genes at an accelerated pace. Gene identification alone, however, is only a preliminary step towards gaining control over the associated diseases and conditions. Methods to manipulate the expression of these newly identified genes are needed as well.

Currently, several general methods have been developed to regulate and control gene expression at either the transcriptional or translational steps. Each of these methods suffers from significant drawbacks.

A. Global transcription and translation regulators

One means of regulating gene expression is to use chemicals that alter the expression of all genes within a cell, tissue, or organism. For example, cycloheximide blocks the peptidyl transferase reaction on eukaryotic ribosomes and acts as a general inhibitor of translation (*i.e.*, the translation of all genes within treated cells is inhibited). Likewise, α-amantin globally blocks mRNA synthesis by binding to eukaryotic RNA polymerase II. Furthermore, actinomycin D is capable of blocking RNA synthesis by intercalating into guanine-cytosine base pairs and disrupting transcription; netropsin and distamycin A block transcription by binding to DNA and blocking RNA polymerase; and acridines, such as proflavine, inhibit RNA synthesis by blocking the formation of the DNA/RNA polymerase complex. Because these chemicals prevent the expression of all genes, any prolonged treatment results in the loss of critical factors needed to maintain the cells, leading to

irreparable damage or cell death (*e.g.*, α-amantin was originally identified as a potent poison from the mushroom *Amanita phalloides*; Wieland and Faulstich, Crit. Rev. B. 5: 185 [1978]). To overcome these drawbacks, methods of regulating the expression of specific genes or gene families must be developed.

B. Regulation of signal transduction pathways

One means of regulating gene expression is to activate or repress the signal transduction pathways that are responsible for regulating gene transcription. By activating or inhibiting important steps in the pathways (*e.g.*, binding of signalling molecules to receptors, entry of signalling molecules into cells or nuclei, covalent modification of enzymes, or release or sequestration of ions from organelles), gene expression can be activated or repressed. For example, pain relievers such as aspirin and ibuprofen inhibit the enzymatic production of prostaglandins and result in decreased swelling and inflammation brought about by the signalling pathways normally initiated by the prostaglandins.

Unfortunately, the regulation of signal transduction pathways is not a viable means of treating many diseases and conditions. Most pathways have not been sufficiently characterized to rationally develop means of regulating expression and treating disease while avoiding unwanted side-effects. For example, many signal transduction pathways regulate a variety of genes in a variety of different cell types. Thus, in an attempt to shut off a gene responsible for a given disease, the pathway may also down-regulate other genes responsible for critical metabolic processes in the cells. Also, many signalling pathways are redundant (*i.e.*, more than one pathway controls the down-stream regulatory event). Thus, by activating or repressing one pathway, another may compensate and confound the attempt at controlling gene expression. Furthermore, many signal transduction pathways cross-talk (*i.e.*, share similar components and co-regulate one another). Thus the regulation of one pathway may result in the undesired regulation of other known, and yet unidentified, pathways. By inhibiting or activating a given step within a pathway, a range of known or unknown side-effects can occur. For example, prostaglandin signalling is involved not only in inflammatory responses, but is also believed to be involved in platelet aggregation, the sleep-wake cycle, some aspects of vision, luteolysis, and any number of yet unidentified physiological effects. Thus, in general, the regulation of signal transduction pathways provides a too broad and unpredictable means for controlling gene expression.

C. Gene therapy

With the development of gene therapy techniques, it has become possible to replace or insert genes of interest into organisms. In theory, overactively expressed or mutated genes can be replaced by "normal" copies. Also, genes can be linked to controllable promoter elements (*i.e.*, a promoter that can be turned on or off by administration of appropriate signalling compounds) and can be placed into target cells. For example, the gene for a desired transcription factor could be placed under the control of such an inducible/repressible promoter. Using this technique, gene families that are activated or repressed by the transcription factor can be coordinately regulated by the administration of the appropriate signalling compounds. These transcription factors can be wild-type (*i.e.*, to directly activate or repress a gene), mutants with DNA binding capability but altered active sites (*i.e.*, to compete with the cell's natural transcription factors for binding to gene enhancers), or mutants with wild-type heterodimerization domains but altered active sites or DNA binding sites (*i.e.*, to bind to the cell's natural transcription factors and prevent it from binding to enhancers and regulating gene expression).

Unfortunately, gene therapy techniques, as described above, are only in their initial stages of development. There are still significant problems to overcome, such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions (Verma and Somia, Nature 389, 239 [1997]). Even if these technologies eventually become widely available, they will be extremely complex, time-consuming, and unpredictable.

The art remains in need of means for regulating gene expression to control and treat human diseases such as cancer and viral infections. Such an approach should repress or activate specific genes or gene families without producing harmful side effects.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions relating to oligonucleotides, with high affinity for a target transcription factor, that can be introduced into cells as decoy *cis*-elements to bind the factor and alter gene expression.

In one embodiment, the present invention provides a composition comprising one or more purified nucleic acid molecules that compete with cAMP response element (CRE) enhancer DNA for binding to one or more transcription factors. In some embodiments the nucleic acid molecules comprise DNA, although all nucleic acid molecules (*e.g.*, RNA) are contemplated by the present invention.

In some embodiments the nucleic acid molecules comprise one or more single-stranded ligonucleotides that will hybridize to form a duplex. In preferred embodiments, the single-stranded oligonucleotides comprise one or more palindromic sequences. In particularly preferred embodiments, the single-stranded oligonucleotides comprise SEQ ID NO:2. However, all single-stranded oligonucleotide or oligonucleotides that can compete with cAMP response element (CRE) enhancer DNA for binding to one or more transcription factors are contemplated by the present invention.

In other embodiments, the nucleic acid molecules comprise one or more hairpin-forming single-stranded oligonucleotides. In preferred embodiments, the hairpin-forming single-stranded oligonucleotides comprise SEQ ID NO:3. In other embodiments, the nucleic acid molecule comprises two hairpin-forming oligonucleotides complementary to one another in a manner wherein combining the two hairpin-forming oligonucleotides produces a cruciform structure. In some embodiments, these two hairpin forming oligonucleotides comprise SEQ ID NO:10 and SEQ ID NO:11.

Although not required by the present invention, in some embodiments the nucleic acid molecules contain modified phosphodiester bonds. In some embodiments, these modified phosphodiester bonds are selected from the group consisting of phosphorothioate, phosphoramidite, and methylphosphonate derivatives.

The present invention provides methods for regulating gene transcription in target cells comprising: providing one or more cAMP response element decoys and one or more target cells containing cAMP response element enhancer DNA and one or more transcription factors that associate with the cAMP response element enhancer DNA; and exposing the target cells to the cAMP response element decoys under condition such that the cAMP response element decoys will compete with the cAMP response element enhancer DNA for binding to the one or more transcription factors.

In some embodiments of this method, the cAMP response element decoys comprise DNA, although all nucleic acid (*e.g.*, RNA) molecules are contemplated by the present invention. In other embodiments the cAMP response element decoys comprise one or more single-stranded oligonucleotides that will hybridize to form a duplex. In preferred embodiments, the single-stranded oligonucleotides comprise one or more palindromic sequences. In particularly preferred embodiments, the single-stranded oligonucleotides comprise SEQ ID NO:2. However, all single-stranded oligonucleotide or oligonucleotides

that compete with cAMP response element (CRE) enhancer DNA for binding to one or more transcription factors are contemplated by the present invention.

In other embodiments of this method, the cAMP response element decoys comprise one or more hairpin-forming single-stranded oligonucleotides. In preferred embodiments, the hairpin-forming single-stranded oligonucleotides comprise SEQ ID NO:3. In other embodiments, the cAMP response element decoys comprises two hairpin-forming oligonucleotides complementary to one another in a manner wherein combining the two hairpin-forming oligonucleotides produces a cruciform structure. In some embodiments, these two hairpin forming oligonucleotides comprise SEQ ID NO:10 and SEQ ID NO:11.

Although not required by the present invention, in some embodiments the cAMP response element decoys contain modified phosphodiester bonds. In some embodiments, these modified phosphodiester bonds are selected from the group consisting of phosphorothioate, phosphoramidite, and methylphosphonate derivatives.

In certain embodiments of the present method, the target cells comprise cancer cells.

In some embodiments, the method of exposing of the target cells to the cAMP response element decoys is selected from the group consisting of injection, intravenous direct exposure, oral intake, transfection, transgenic expression, time-release pellet, and micropump administration, although all methods of exposure are contemplated by the present invention.

The present invention further provides methods for regulating cancer cell proliferation *in vivo* comprising: providing one or more cAMP response element decoys and one or more target cells containing cAMP response element enhancer DNA and one or more transcription factors that associate with the cAMP response element enhancer DNA; and exposing the target cells to the cAMP response element decoys under condition such that the cAMP response element decoys will compete with the cAMP response element enhancer DNA for binding to the one or more transcription factors.

In some embodiments of this method, the cAMP response element decoys comprise DNA, although all nucleic acid molecules are contemplated by the present invention. In other embodiments the cAMP response element decoys comprise one or more single-stranded oligonucleotides that will hybridize to form a duplex. In preferred embodiments, the single-stranded oligonucleotides comprise one or more palindromic sequences. In particularly preferred embodiments, the single-stranded oligonucleotides comprise SEQ ID NO:2. However, all single-stranded oligonucleotide or oligonucleotides that can compete with cAMP

response element (CRE) enhancer DNA for binding to one or more transcription factors are contemplated by the present invention.

In other embodiments of this method, the cAMP response element decoys comprise one or more hairpin-forming single-stranded oligonucleotides. In preferred embodiments, the hairpin-forming single-stranded oligonucleotides comprise SEQ ID NO:3. In other embodiments, the cAMP response element decoys comprises two hairpin-forming oligonucleotides complementary to one another in a manner wherein combining the two hairpin-forming oligonucleotides produces a cruciform structure. In some embodiments, these two hairpin forming oligonucleotides comprise SEQ ID NO:10 and SEQ ID NO:11.

Although not required by the present invention, in some embodiments the cAMP response element decoys contain modified phosphodiester bonds. In some embodiments, these modified phosphodiester bonds are selected from the group consisting of phosphorothioate, phosphoramidite, and methylphosphonate derivatives.

In some embodiments, the method of exposing of the target cells to the cAMP response element decoys is selected from the group consisting of injection, intravenous direct exposure, oral intake, transfection, transgenic expression, time-release pellet, and micropump administration, although all methods of exposure are contemplated by the present invention.

DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram showing the transitions and binding affinities of transcription factors to linear duplex and hairpin cruciform oligonucleotides.

Figure 2 shows a photograph of CRE-oligonucleotide decoy inhibition of CRE(DNA)-protein complex formation.

Figure 3 shows data obtained from thin-layer chromatography of CRE-oligonucleotide decoy inhibition of CRE-directed transcription.

Figure 4 shows CRE-oligonucleotide decoy inhibition of cancer cell growth *in vitro* in several different cell lines.

Figure 5 shows CRE-oligonucleotide decoy inhibition of tumor growth *in vivo* by measuring tumor volume.

Figure 6 shows CRE-oligonucleotide decoy induction of apoptosis as demonstrated by: A) whole cell morphology; and B) nuclear morphology.

Figure 7 shows Bcl-2 protein levels in CRE-oligonucleotide treated cells and control cells as measured by Western blot analysis.

Figure 8 shows the effect of the CRE-oligonucleotides of the present invention interfered on both basal and cAMP-induced transcription of an exogenously supplied CRE-containing gene.

Figure 9 shows the effect of CRE palindrome oligonucleotide treatment on the reduction in the mRNA levels of the catalytic (Cα) and regulatory (RIα) subunits of cAMP-dependent protein kinase (PKA) and PEPCK in MCF7 breast cancer cells.

Figure 10 shows the effect of CRE-oligonucleotide treatment on AP-1 binding.

Figure 11 shows cellular uptake and stability of CRE-oligonucleotides showing A) cellular incorporation of oligonucleotide; B) non-denaturing polyacrylamide gel electrophoresis of cell-incorporated CRE-oligonucleotide; and C) UV melting study results.

Figure 12 shows A) gel retardation assay data of nuclear extracts from cells treated with the 24-mer CRE oligonucleotide; and B) Western blot analysis for CREB protein in untreated, CRE oligo treated, and control oligo treated cells.

Figure 13 shows Northern Blot assay data of the effects of CRE-decoy on TPA-inducible mRNA level of *c-fos*.

DEFINITIONS

To facilitate an understanding of the invention, a number of terms are defined below.

The term "biologically active," as used herein, refers to a protein or other biologically active molecules (*e.g.*, catalytic RNA) having structural, regulatory, or biochemical functions of a naturally occurring molecule.

The term "agonist," as used herein, refers to a molecule which, when interacting with an biologically active molecule, causes a change (*e.g.*, enhancement) in the biologically active molecule, which modulates the activity of the biologically active molecule. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules which bind or interact with biologically active molecules. For example, agonist can alter the activity of gene transcription by interacting with RNA polymerase directly or through a transcription factor.

The terms "antagonist" or "inhibitor," as used herein, refer to a molecule which, when interacting with a biologically active molecule, blocks or modulates the biological activity of the biologically active molecule. Antagonists and inhibitors may include proteins, nucleic acids, carbohydrates, or any other molecules which bind or interact with biologically active molecules. Inhibitors and antagonists can effect the biology of entire cells, organs, or organisms (*e.g.*, an inhibitor that slows tumor growth).

The term "modulate," as used herein, refers to a change or an alteration in the biological activity of a biologically active molecule. Modulation may be an increase or a decrease in activity, a change in binding characteristics, or any other change in the biological, functional, or immunological properties of biologically active molecules.

As used herein, the term "nucleic acid molecule" refers to any nucleic acid containing molecule including, but not limited to DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor (*e.g.*, c-myc). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences."

Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (*e.g.*, mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (*i.e.*, via the enzymatic action of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be regulated at many stages in the process. "Up-regulation" or "activation" refers to regulation that increases the production of gene expression products (*i.e.*, RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (*e.g.*, transcription factors) that are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

As used herein, the term "pleiotropic activator" refers to activators that function on a multiplicity of different genes and have a multiplicity of different effects.

Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3' flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional

properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides or polynucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides or polynucleotide, referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide or polynucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

As used herein, the terms "an oligonucleotide having a nucleotide sequence encoding a gene" and "polynucleotide having a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide or polynucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention

may contain endogenous enhancers/promoters, splice junctions, interv ning sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

As used herein, the term "oligonucleotide," refers to a short length of single-stranded polynucleotide chain. Oligonucleotides are typically less than 100 residues long (*e.g.*, between 15 and 50), however, as used herein, the term is also intended to encompass longer polynucleotide chains. Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by hybridizing to other polynucleotides. Such structures can include, but are not limited to, duplexes, hairpins, cruciforms, bends, and triplexes.

As used herein, the term "transcription factor" refers to proteins that interact with one another and RNA polymerase enzyme to modulate transcription. Transcription factors target genes by recognizing specific DNA regulatory sequences (*e.g.*, enhancers) or other transcription factors. Transcription factors are often referred to as "*trans*-factors" that interact with "*cis*-elements" (*e.g.*, enhancers) because they are typically produced from genes located distantly (trans) from their sites of regulation (cis). Some transcription factors are biologically active only when bound to another copy of itself (*i.e.*, homodimers linked through "homodimerization domains") or to other transcription factors (*i.e.*, heterodimers linked through "heterodimerization domains"). For most transcription factors, specific and distinct regions of the protein mediate DNA binding (*i.e.*, "DNA binding domains") and transcriptional activation (*i.e.*, "activation domains"). The term "CRE transcription factor" refers to transcription factors (*e.g.*, peptides) that recognize and bind to cAMP response elements (*i.e.*, "cAMP response element enhancer DNA") or to proteins bound to such elements. This term encompasses both identified (*e.g.*, CREB) and yet unidentified transcription factors.

As used herein, the term "trans-dominant mutant" refers to transcription factors that compete with wild-type transcription factors (*i.e.*, "transactivators") for binding to enhancer sequences.

As used herein, the terms "decoy" and "transcription factor decoy" refer to molecules that bind to or interact with transcription factors and prevent their binding to native enhancer sequences. Decoys include nucleic acid sequences, including, but not limited to, oligonucleotides that correspond to (*i.e.*, are identical to or essentially identical to) the native

enhancer. Such oligonucleotides include, but are not limited to, single stranded palindromic oligonucleotides comprising one or more repeats of the enhancer sequence, sense and antisense oligonucleotides comprising one or more repeats of the enhancer sequence, oligonucleotides that form hairpin structures such that a duplex binding site for the transcription factor is generated, and one or more oligonucleotides that form a cruciform structure such that one or more binding sites for the transcription factor are generated. The terms "CRE transcription factor decoy" and "cAMP response element decoy" refer to decoys that target transcription factors associated with cAMP response elements.

As used herein, the term "duplex," in reference to oligonucleotides, refers to regions that are double stranded through hybridization of complementary base pairs. The term "hairpin" refers to double-stranded nucleic acid structures formed by base-pairing between regions of the same strand of a nucleic acid molecule. The regions are arranged inversely and can be adjacent or separated by noncomplementary sequence (*i.e.*, thus forming a loop structure or "stem-loop"). The term "cruciform" refers to structures formed in double-stranded nucleic acids by inverted repeats separated by a short sequence. Cruciform structures can be generated through the hybridization of two or more hairpin structures where the hairpin duplex and loop comprise the short sequence separating the inverted repeats. Cruciform structures can comprise one or more nucleic acid molecules.

As used herein, the term "palindrome" refers to regions of nucleic acid in which the sequence of both strands is identical when read in antiparallel directions (*i.e.*, both strands read 5' to 3' or 3' to 5').

As used herein, the term "high affinity" refers to the non-random interaction of a molecule with itself or another molecule. Molecules with affinity for one another will tend to "bind" (*i.e.*, chemically associate through weak or strong chemical interactions) and form a stable complex. For example, a transcription factor will have high affinity for polynucleotide sequences that correspond to its DNA binding domain and low affinity for other nucleic acid sequences.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, etc. (defined *infra*).

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (T. Maniatis *et al.*, Science 236:1237 [1987]). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in prokaryote). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review see, S.D. Voss *et al.*, Trends Biochem. Sci., 11:287 [1986]; and T. Maniatis *et al.*, *supra*). For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells (R. Dijkema *et al.*, EMBO J. 4:761 [1985]). Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1α gene (T. Uetsuki *et al.*, J. Biol. Chem., 264:5791 [1989]; D.W. Kim *et al.*, Gene 91:217 [1990]; and S. Mizushima and S. Nagata, Nuc. Acids. Res., 18:5322 [1990]) and the long terminal repeats of the Rous sarcoma virus (C.M. Gorman *et al.*, Proc. Natl. Acad. Sci. USA 79:6777 [1982]) and the human cytomegalovirus (M. Boshart *et al.*, Cell 41:521 [1985]).

As used herein, the term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (*i.e.*, the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter.

As used herein, the term "cAMP response element" or "CRE" refers to enhancer sequences that interact with transcription factors which mediate signal transduction involving cAMP. A consensus sequence has been described for CRE enhancers, comprising 5'-TGACGTCA-3' (SEQ ID NO:1). However, CREs from many genes that deviate from this sequence have been identified (Roesler *et al.*, *supra*). Such sequences include, but are not limited to, TTACGTCA (SEQ ID NO:4) (Short *et al.*, J. Biol. Chem. 261, 9721 [1986]), TGACGTCT (SEQ ID NO:5) (Tsukada *et al.*, J. Biol. Chem. 262, 8743 [1987]), TGACGTAG (SEQ ID NO:6) (VanBeveren *et al.*, Cell 32, 1241 [1983]), and CTGCGTCA (SEQ ID NO:7) (Comb *et al.*, Nature 323, 353 [1986]). Genes that have CREs are referred to as "cAMP-sensitive genes."

As used herein, the term "Nucleic acid molecules that compete with response element enhancer DNA for binding to transcription factors" refers to any nucleic acid molecule with affinity for a transcription factor DNA binding site or otherwise interacts with the transcription factor to prevent or reduce binding to native enhancer sequences. Thus, such molecules are defined functionally, rather than strictly structurally. In this regard, such functionality is readily testable in the assays described herein. Specifically, the assay described in Example 1 below provides a convenient test format for screening candidate molecules and thereby identifying competing molecules. Additionally, there are a variety of computer programs and services available for comparing sequence and structural information about oligonucleotides that may be used to identify candidates for submitting to the functional screening methods described in Example 1. Such candidate molecules typically comprise one or more duplex portions, including but not limited to linear duplex, hairpin, and cruciform structures. In some embodiments, these molecules comprise the consensus sequence for a given transcription factor (*e.g.*, the CRE consensus sequence 5'-TGACGTCA-3'). Additionally, molecules similar to the consensus sequence (*i.e.*, molecules that have one or a few base substitutions, deletions, or additions from the known response element and that retain their palindromic or secondary structural characteristics) provide effective decoys, with potency varying by gene, cell type, and species (*e.g.*, duplex forming oligonucleotides that are sufficiently similar in sequence to the consensus sequence or any known response element can be used as decoys). However, regardless of sequence, any oligonucleotide that contains sufficient structure to exhibit affinity for the DNA binding site of the targeted transcription factor will find use with, and is contemplated by, the present invention.

As used herein, the term "CRE-transcription factor complex" refers to the collection of proteins (*e.g.*, transcription factors) involved in regulating gene expression through a cAMP response element. Such proteins include known factors (*e.g.*, CREB) and yet unidentified factors.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (J. Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York [1989], pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp *Bam*HI/*Bcl*I restriction fragment and directs both termination and polyadenylation (J. Sambrook, *supra*, at 16.6-16.7).

Eukaryotic expression vectors may also contain "viral replicons "or "viral origins of replication." Viral replicons are viral DNA sequences which allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors which contain either the SV40 or polyoma virus origin of replication replicate to high "copy number" (up to 10^4 copies/cell) in cells that express the appropriate viral T antigen. Vectors which contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at "low copy number" (~100 copies/cell).

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For

example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

The art knows well that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions which promote hybridization under conditions of high stringency (*e.g.*, increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.) (see definition below for "stringency").

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

A gene may produce multiple RNA species which are generated by differential splicing of the primary RNA transcript. cDNAs that are splice variants of the same gene will contain regions of sequence identity or complete homology (representing the presence of the same exon or portion of the same exon on both cDNAs) and regions of complete non-identity (for example, representing the presence of exon "A" on cDNA 1 wherein cDNA 2 contains exon "B" instead). Because the two cDNAs contain regions of sequence identity they will both hybridize to a probe derived from the entire gene or portions of the gene containing sequences found on both cDNAs; the two splice variants are therefore substantially homologous to such a probe and to each other.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe which can hybridize (*i.e.*, it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids. A single molecule that contains pairing of complementary nucleic acids within its structure is said to be "self-hybridized."

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*See e.g.,* Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m.

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Qβ replicase, MDV-1 RNA is the specific template for the replicase (D.L. Kacian *et al.*, Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other nucleic acid will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (M. Chamberlin *et al.*, Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (D.Y. Wu and R. B. Wallace, Genomics 4:560 [1989]). Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences (H.A. Erlich (ed.), *PCR Technology*, Stockton Press [1989]).

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labelled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target," refers to the region of nucleic acid bounded by the primers. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis U.S. Patent Nos. 4,683,195 4,683,202, and 4,965,188, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ^{32}P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

As used herein, the terms "PCR product," "PCR fragment," and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR

steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or m re target sequences.

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used herein, the term "RT-PCR" refers to the replication and amplification of RNA sequences. In this method, reverse transcription is coupled to PCR, most often using a one enzyme procedure in which a thermostable polymerase is employed, as described in U.S. Patent No. 5,322,770, herein incorporated by reference. In RT-PCR, the RNA template is converted to cDNA due to the reverse transcriptase activity of the polymerase, and then amplified using the polymerizing activity of the polymerase (*i.e.*, as in other PCR methods).

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "antisense" is used in reference to DNA or RNA sequences which are complementary to a specific DNA or RNA sequence (*e.g.*, mRNA). Included within this definition are antisense RNA ("asRNA") molecules involved in gene regulation by bacteria. Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a coding strand. Once introduced into an embryo, this transcribed strand combines with natural mRNA produced by the embryo to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand.

The terms "in operable combination," "in operable order," and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids as nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNA s which encode a multitude of proteins. However, isolated nucleic acid encoding a given protein includes, by way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide or polynucleotide may be double-stranded).

As used herein, a "portion of a chromosome" refers to a discrete section of the chromosome. Chromosomes are divided into sites or sections by cytogeneticists as follows: the short (relative to the centromere) arm of a chromosome is termed the "p" arm; the long arm is termed the "q" arm. Each arm is then divided into 2 regions termed region 1 and region 2 (region 1 is closest to the centromere). Each region is further divided into bands. The bands may be further divided into sub-bands. For example, the 11p15.5 portion of human chromosome 11 is the portion located on chromosome 11 (11) on the short arm (p) in the first region (1) in the 5th band (5) in sub-band 5 (.5). A portion of a chromosome may be "altered;" for instance the entire portion may be absent due to a deletion or may be rearranged (*e.g.*, inversions, translocations, expanded or contracted due to changes in repeat regions). In the case of a deletion, an attempt to hybridize (*i.e.*, specifically bind) a probe homologous to a particular portion of a chromosome could result in a negative result (*i.e.*, the probe could not bind to the sample containing genetic material suspected of containing the missing portion of the chromosome). Thus, hybridization of a probe homologous to a

particular portion of a chromosome may be used to detect alterations in a portion of a chromosome.

The term "sequences associated with a chromosome" means preparations of chromosomes (*e.g.*, spreads of metaphase chromosomes), nucleic acid extracted from a sample containing chromosomal DNA (*e.g.*, preparations of genomic DNA); the RNA which is produced by transcription of genes located on a chromosome (*e.g.*, hnRNA and mRNA) and cDNA copies of the RNA transcribed from the DNA located on a chromosome. Sequences associated with a chromosome may be detected by numerous techniques including probing of Southern and Northern blots and *in situ* hybridization to RNA, DNA or metaphase chromosomes with probes containing sequences homologous to the nucleic acids in the above listed preparations.

As used herein the term "coding region" when used in reference to structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

As used herein, the term "structural gene" refers to a DNA sequence coding for RNA or a protein. In contrast, "regulatory genes" are structural genes which encode products which control the expression of other genes (*e.g.*, transcription factors).

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample. In another example, recombinant polypeptides are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein to indicate that a protein does not contain amino acid residues encoded by vector sequences; that is the native protein contains only those amino acids found in the protein as it occurs in nature. A native protein may be produced by recombinant means or may be isolated from a naturally occurring source.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

The term "Southern blot," refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size followed by transfer of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (J. Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58 [1989]).

The term "Northern blot," as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (J. Sambrook, J. *et al.*, supra, pp 7.39-7.52 [1989]).

The term "Western blot" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an antigen of interest. The binding of the antibodies may be detected by various methods, including the use of radiolabelled antibodies.

The term "antigenic determinant" as used herein refers to that portion of an antigen that makes contact with a particular antibody (*i.e.*, an epitope). When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce

the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (*i.e.*, the "immunogen" used to elicit the immune response) for binding to an antibody.

The terms "specific binding" or specifically binding" when used in reference to the interaction of an antibody and a protein or peptide means that the interaction is dependent upon the presence of a particular structure (*i.e.*, the antigenic determinant or epitope) on the protein; in other words the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A," the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labelled "A" and the antibody will reduce the amount of labelled A bound to the antibody.

The term "transgene" as used herein refers to a foreign gene that is placed into an organism by introducing the foreign gene into newly fertilized eggs or early embryos. The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) which is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene does not reside in the same location as does the naturally-occurring gene.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

Embryonal cells at various developmental stages can be used to introduce transgenes for the production of transgenic animals, often referred to as "gene therapy." Different methods are used depending on the stage of development of the embryonal cell. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2 picoliters (pl) of DNA solution. The use of zygotes as a target for gene transfer has a major

advantage in that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442 [1985]). As a consequence, all cells of the transgenic animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Micro-injection of zygotes is the preferred method for incorporating transgenes in practicing the invention. U.S. Patent No. 4,873191 describes a method for the micro-injection of zygotes; the disclosure of this patent is incorporated herein in its entirety.

Retroviral infection can also be used to introduce transgenes into animals. The developing embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Janenich, Proc. Natl. Acad. Sci. USA 73:1260-1264 [1976]). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan *et al.*, in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. [1986]). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (D. Jahner *et al.*, Proc. Natl. Acad Sci. USA 82:6927-693 [1985]). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al.*, EMBO J. 6:383-388 [1987]). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (D. Jahner *et al.*, Nature 298:623-628 [1982]). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of cells which form the transgenic animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germline, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner *et al.*, supra [1982]). Additional means of using retroviruses or retroviral vectors to create transgenic animals known to the art involves the micro-injection of retroviral particles or mitomycin C-treated cells producing retrovirus into the perivitelline space of fertilized eggs or early embryos (PCT International Application WO 90/08832 [1990], and Haskell and Bowen, Mol. Reprod. Dev., 40:386 [1995]).

A third type of target cell for transgene introduction is the embryonal stem (ES) cell. ES cells are obtained by culturing pre-implantation embryos *in vitro* under appropriate conditions (Evans *et al.*, Nature 292:154-156 [1981]; Bradley *et al.*, Nature 309:255-258

[1984]; Gossler *et al.*, Proc. Acad. Sci. USA 83:9065-9069 [1986]; and Robertson *et al.*, Nature 322:445-448 [1986]). Transgenes can be efficiently introduced into the ES cells by DNA transfection by a variety of methods known to the art including calcium phosphate co-precipitation, protoplast or spheroplast fusion, lipofection and DEAE-dextran-mediated transfection. Transgenes may also be introduced into ES cells by retrovirus-mediated transduction or by micro-injection. Such transfected ES cells can thereafter colonize an embryo following their introduction into the blastocoel of a blastocyst-stage embryo and contribute to the germ line of the resulting chimeric animal (for review, *See*, Jaenisch, Science 240:1468-1474 [1988]). Prior to the introduction of transfected ES cells into the blastocoel, the transfected ES cells may be subjected to various selection protocols to enrich for ES cells which have integrated the transgene assuming that the transgene provides a means for such selection. Alternatively, the polymerase chain reaction may be used to screen for ES cells which have integrated the transgene. This technique obviates the need for growth of the transfected ES cells under appropriate selective conditions prior to transfer into the blastocoel.

The terms "overexpression" and "overexpressing" and grammatical equivalents, are used in reference to levels of mRNA to indicate a level of expression approximately 3-fold higher than that typically observed in a given tissue in a control or non-transgenic animal. Levels of mRNA are measured using any of a number of techniques known to those skilled in the art including, but not limited to Northern blot analysis. Appropriate controls are included on the Northern blot to control for differences in the amount of RNA loaded from each tissue analyzed (*e.g.*, the amount of 28S rRNA, an abundant RNA transcript present at essentially the same amount in all tissues, present in each sample can be used as a means of normalizing or standardizing the mRNA-specific signal observed on Northern blots). The amount of mRNA present in the band corresponding in size to the correctly spliced transgene RNA is quantified; other minor species of RNA which hybridize to the transgene probe are not considered in the quantification of the expression of the transgenic mRNA.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells which have taken up foreign DNA but have failed to integrate this DNA.

The term "calcium phosphate co-precipitation" refers to a technique for the introduction of nucleic acids into a cell. The uptake of nucleic acids by cells is enhanced when the nucleic acid is presented as a calcium phosphate-nucleic acid co-precipitate. The original technique of Graham and van der Eb (Graham and van der Eb, Virol., 52:456 [1973]), has been modified by several groups to optimize conditions for particular types of cells. The art is well aware of these numerous modifications.

As used herein, the term "selectable marker" refers to the use of a gene which encodes an enzymatic activity that confers the ability to grow in medium lacking what would otherwise be an essential nutrient (*e.g.* the *HIS3* gene in yeast cells); in addition, a selectable marker may confer resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be "dominant"; a dominant selectable marker encodes an enzymatic activity which can be detected in any eukaryotic cell line. Examples of dominant selectable markers include the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the *neo* gene) which confers resistance to the drug G418 in mammalian cells, the bacterial hygromycin G phosphotransferase (*hyg*) gene which confers resistance to the antibiotic hygromycin and the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the *gpt* gene) which confers the ability to grow in the presence of mycophenolic acid. Other selectable markers are not dominant in that there use must be in conjunction with a cell line that lacks the relevant enzyme activity. Examples of non-dominant selectable markers include the thymidine kinase (*tk*) gene which is used in conjunction with *tk*⁻ cell lines, the CAD gene which is used in conjunction with CAD-deficient cells and the mammalian hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene which is used in conjunction with *hprt*⁻ cell lines. A review of the use of selectable markers in mammalian cell lines is provided in Sambrook, J. *et al.*, *Molecular Cloning: A*

Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp.16.9-16.15.

As used herein, the term "signal transduction" refers to the process of transferring information from a chemical signal (*e.g.*, a hormone, a growth factor, or neurotransmitter) into a cell and along an intracellular chain of signalling molecules (*i.e.*, a "signal transduction pathway") to stimulate the appropriate cellular response (*e.g.*, activation or repression of gene expression). For example, an extracellular signal can bind to a cell membrane receptor and activate the enzyme adenyl cyclase, leading to an increase in intracellular cAMP concentrations. The increase in cAMP concentrations can activate other intracellular proteins that can eventually lead to the binding of an active transcription factor to CRE elements and alter gene expression of CRE-sensitive genes.

As used herein, the term "intercalation" refers to the process of interposing or inserting something between two or more objects. For example, ethidium bromide can intercalate between nucleotides within a DNA molecule, while actinomycin D can intercalate between guanine-cytosine base pairs.

As used herein, the term "phosphodiester bond" refers to the covalent phosphate linkage between residues in a polynucleotide chain.

As used herein, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*.

As used, the term "eukaryote" refers to organisms distinguishable from "prokaryotes." It is intended that the term encompass all organisms with cells that exhibit the usual characteristics of eukaryotes, such as the presence of a true nucleus bounded by a nuclear membrane, within which lie the chromosomes, the presence of membrane-bound organelles, and other characteristics commonly observed in eukaryotic organisms. Thus, the term includes, but is not limited to such organisms as fungi, protozoa, and animals (*e.g.*, humans).

As used herein, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments can consist of, but are not limited to, test tubes and cell culture. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

As used herein, the term "differentiation" refers to the expression and manifestation of the fate of a cell. The term "apoptosis" refers to the programmed (*i.e.*, genetically controlled) death of a cell. Apoptosis is characterized by loss of cell junctions, loss of micovilli, condensed cytoplasm, margination of nuclear chromatin into discrete masses, compacting of mitochondria and ribosomes, dilation of the endoplasmic reticulum, and break-up of cells into several membrane bound bodies (*i.e.*, apoptotic bodies).

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

A "composition comprising a given polynucleotide sequence" as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise an aqueous solution. Compositions comprising polynucleotide sequences that are partially or completely complementary to an enhancer element (*e.g.*, SEQ ID NO:1) may be employed as transcription factor decoys.

The term "sample" as used herein is used in its broadest sense. A sample suspected of containing a human chromosome or sequences associated with a human chromosome may comprise a cell, chromosomes isolated from a cell (*e.g.*, a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern blot analysis), RNA (in solution or bound to a solid support such as for Northern blot analysis), cDNA (in solution or bound to a solid support) and the like. A sample suspected of containing a protein may comprise a cell, a portion of a tissue, an extract containing one or more proteins and the like.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions relating to oligonucleotides, with high affinity for a target transcription factor, that can be introduced into cells as decoy *cis*-elements to bind the factor and alter gene expression. Specifically, the present invention provides nucleic acid molecules that compete with cAMP response element (CRE) enhancers for binding to transcription factors. These nucleic acid molecules were shown to function *in*

vitro and *in vivo* as inhibitors of tumor cell growth, without affecting the growth of non-cancerous cells. The present invention provides a powerful new means of combatting cancers by regulating the expression of cAMP-sensitive genes.

A. **Enhancers and Transcription Factors**

Eukaryotic transcription is regulated by the interplay of various protein factors at promoters (Maniatis *et al.*, Science 236, 1237 [1987]). RNA polymerase binds to the promoter and catalyzes the synthesis of RNA from the DNA template. The binding of RNA polymerase and its activity can be regulated by the presence or absence of other protein regulators (*i.e.*, transcription factors). Some transcription factors (*e.g.*, activators or repressors) bind to specific DNA sequences called enhancers. Enhancer are typically located 5' of the gene they regulate, but can be found within the gene itself, 3' of the gene, distantly 5' of the gene, or even on other portions of the chromosome. Some transcription factors do not directly bind to enhancer sequences, but are associated with other proteins that do.

The displacement of transcription factors from their enhancer binding sites offers a means of regulating gene expression. For example, it has been shown that prokaryotic repressors can function as negative regulators of eukaryotic promoters (Hu and Davidson, Cell 48: 555 [1987]). This observation suggests that displacement of activating proteins might provide a general strategy for gene-specific repression in eukaryotes. Several approaches have been undertaken to control eukaryotic gene expression through such displacement, although none have found great success.

In one approach, trans-dominant mutants are generated that interfere with the function of transactivators. Mutants are generated that retain the ability to bind to *cis*-regulatory DNA sequences but that have dysfunctional transcriptional activation domains. These mutant transcription factors compete with their functional, wild-type counterparts for binding to the enhancer sequences and prevent the activation or repression or the target gene. While this strategy has been successful, *in vitro* (*See e.g.*, Friedman *et al.*, Nature 335: 452 [1988]), the generation of such mutants is not always possible. The transcription factor must be well characterized such that the activation domain(s) are identified and can be mutated. Also, even with sufficient knowledge to generate such mutants, time consuming, expensive, and difficult gene therapy procedures would be required to express these proteins *in vivo*.

In another approach, promoter competition is utilized whereby plasmids containing *cis*-acting elements, in common with the targeted gene, are introduced in high copy number into

cells (Wang and Calame, Cell 47: 241 [1986]). At high copy number, a majority of the transcripti n factors can be competitively bound away from the natural enhancer sequences with gene expression accordingly regulated. Because these plasmids must be maintained uniformly in large numbers of cells, this approach has also been limiting.

Another approach used oligonucleotides to form triple helices with enhancer elements. Pyrimidine oligonucleotides were found to bind with sequence-specific dependence to homopurine sites in duplex DNA by triple helix formation and had sufficient specificity and affinity to compete with site-specific DNA binding proteins for occupancy of overlapping target sites (Maher III *et al.*, Science 245, 725 [1989]). However, such oligonucleotide-directed triple helix formation has not been shown in cells *in vitro* or *in vivo*.

B. Competition with the CRE enhancer

The approach of the present invention used oligonucleotides, modified to facilitate entry into cells, that compete with the native cellular cAMP response element (CRE) enhancers for binding to transcription factors. This decoy approach proved successful *in vivo* and *in vitro* in regulating gene expression.

The transcription factor decoys of the present invention are recognized and bound by transcription factors such that the factors can no longer bind to native response elements and regulate gene expression. In some embodiments of the present invention, decoys comprise one or more duplex nucleic acid structures. These structures are recognized by the DNA binding domain of the target transcription factors. The present invention is not intended to be limited to decoys with duplex structures however, as any nucleic acid structure that binds to the DNA binding domains is contemplated. In some embodiments of the present invention, the decoys comprise the consensus sequence for the targeted transcription factor. A consensus sequence is identified as the sequence that, on average (*i.e.*, in the most genes studied thus far or in binding affinity studies), binds with the highest affinity to its associated transcription factor(s). However, the decoys of the present invention are not limited to sequences comprising the consensus sequence. A variety of enhancers, with sequences slightly divergent from a consensus sequence, are often known to bind to the associated transcription factor. The present invention contemplates decoys comprising sequences from such known enhancers. The present invention further contemplates decoys comprising sequences similar to a consensus sequence and other known enhancers. Any decoy that has

affinity for the target transcription factor(s) is suitable for use as a decoy and is contemplated by the present invention.

The CRE, 5'-TGACGTCA-3' (SEQ ID NO:1), has been described as the consensus sequence for the *cis*-element that directs cAMP-induced gene transcription (Roesler *et al.*, J. Biol. Chem. 263, 9063 [1988]). The CRE-transcription factor complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes. However, a CRE decoy oligonucleotide has never been described previously. It was not clear that such an oligonucleotide could be generated in a manner that would effectively compete with the natural CRE enhancers to regulate gene expression. Because the identity of the CRE transcription factor complex or complexes has not been thoroughly characterized, the effect of a given oligonucleotide decoy could not be predicted. For example, it is possible that sequences flanking the CRE enhancer are required for optimal transcriptional regulation by CRE transcription factors in a given gene. If these sequences are not included in the decoy oligonucleotide, the decoy may not effectively compete with the native enhancer for binding to the transcription factor complex. The exact sequence needed for any given gene may vary, making prediction impossible. Therefore, it was not predictable that CRE oligonucleotide decoys would work.

Furthermore, even if such a decoy oligonucleotide worked efficiently, it was not clear how the modification of transcription would affect the targeted cells. Because there are many cAMP-regulated genes and because they are ubiquitous in all cell types, it was feared that the use of CRE decoys would be detrimental to cells and organisms.

Surprisingly, the present invention demonstrated that duplex, hairpin, and cruciform oligonucleotides containing the core CRE consensus sequence (*i.e.*, decoys), effectively regulated gene transcription in a wide variety of cell types. Even more surprisingly, the present invention demonstrated that cancer cell growth was inhibited by CRE decoy oligonucleotides, without adversely affecting non-cancerous cells.

The CRE decoy oligonucleotides of the present invention comprise sequences that contain one or more CRE binding sites. In some embodiments, the decoys comprise oligonucleotides containing the CRE consensus sequence (*i.e.*, 5'-TGACGTCA-3'; SEQ ID NO:1), although any sequence with affinity for CRE transcription factors is contemplated by the present invention. Such sequences include, but are not limited to, TTACGTCA (SEQ ID NO:4) (Short *et al.*, J. Biol. Chem. 261, 9721 [1986]), TGACGTCT (SEQ ID NO:5) (Tsukada *et al.*, J. Biol. Chem. 262, 8743 [1987]), TGACGTAG (SEQ ID NO:6)

(VanBeveren *et al.*, Cell 32, 1241 [1983]), CTGCGTCA (SEQ ID NO:7) (Comb *et al.*, Nature 323, 353 [1986]), TGCGTCA (SEQ ID NO:13), and TGGCGTAG (SEQ ID NO:14) (Kwast-Welfeld *et al.*, Cellular and Molecular Biology Research 39, 231 [1993]). As is clear from these other enhancers, nucleic acid sequences with one or more bases different from the consensus sequence can still be recognized by the transcription factors. Thus a range of sequences can be effectively employed as decoys. By selecting sequences sufficiently divergent from the consensus sequence, decoys can be generated with varying affinities (*i.e.*, potencies).

Furthermore, studies have shown that some cAMP-inducible gene promoters require an additional conserved sequence 3' of the CRE sequence for optimal binding and maximal responsiveness (Kwast-Welfeld *et al.*, *supra*). Therefore, in some embodiments, the present invention contemplates the addition of one or more such flanking sequences 3' of the CRE element, if desired, to optimize decoy potency. Such flanking sequences are typically within six nucleotides of the core CRE and comprise sequences including, but not limited to, GAGA, GAAG, GAGG, GAGC, GGGAG, GGCC, GGAGC, GGGAA, CAGC, GCAG, AGAG, and GAGTA.

In some embodiments, the oligonucleotides of the present invention are synthesized with modified phosphodiester bonds, including, but not limited to phosphorothioate, phosphoramidite, or methylphosphonate derivatives. However, the present invention is not limited to the use of oligonucleotides with modified phosphodiester bonds. The modified oligonucleotides can be synthesized in large amounts and are relatively resistant to nucleases (Zon, Pharm. Res. 5, 539 [1988]; and Agrawal *et al.*, Proc. Natl. Acad. Sci. 85, 7079 [1988]). Because of their increased cell permeability and stability, such compounds have been used as mRNA antisense agents (Crooke, Annu. Rev. Pharmacol. Toxicol. 32, 329 [1992]; and Roush, Science 276, 1192 [1997]). However, unlike the mRNA antisense applications, the present invention takes advantage of these features to provide a means for directly targeting transcription factors rather than mRNA. Furthermore, the present invention provides novel methods and compositions for globally controlling the expression of genes that are regulated through CREs, unlike the antisense method, which only target mRNA for one specific gene product. Also, unlike the oligonucleotides of the present invention, mRNA antisense molecules sometimes trigger dangerous side-effects in animals such as extreme immune responses, blood clotting, cardiovascular problems, and even death (Gura, Science 270: 577 [1995]). Additionally, antisense molecules directed to mRNA require sequence knowledge of

the gene to be regulated. In the present invention, only the sequence of the response element is needed (*i.e.*, knowing the sequence of a response element allows for the regulation of both identified and unidentified genes that are transcriptionally regulated by the element).

In some embodiments of the present invention, the oligonucleotides were palindromic *cis*-transcription elements comprising a synthetic single-stranded oligonucleotide composed of the CRE *cis*-element that self-hybridized to form a duplex. When introduced into cells, these oligonucleotides acted as decoys for the CRE transcription factors and interfered with the *cis*-element-directed transcription. While the present invention is not limited to any particular mechanism, it is known that perfect palindromes are capable of forming strong hairpin structures. Such structures may be formed by the palindromic decoys, facilitating enhanced binding to the target transcription factors.

A similar approach can be used for a *cis*-element that is not palindromic. In this case, two synthetic single-stranded oligonucleotides, each composed of the sense- and antisense-*cis*-element, respectively, in combination can be used as the transcription factor decoy.

In some embodiments of the present invention, these single-stranded oligonucleotides contain multiple copies of the cis-element. In one preferred embodiment, the CRE-palindrome comprises a triplet repeat of the CRE consensus sequence: 5'-TGACGTCATGACGTCATGACGTCA-3' (SEQ ID NO:2).

In other embodiments, synthetic oligonucleotides designed to form hairpin structures and comprising a *cis*-transcription element were used as transcription factor decoys. Recent evidence has indicated that DNA hairpin formation may represent an additional level of transcriptional control. For example, 23-bp synthetic oligonucleotide of human enkephalin gene enhancer has been shown to undergo a reversible conformational change from a duplex to a cruciform structure of two hairpins (McMurray *et al.*, Proc. Natl. Acac. Sci. 88, 666 [1991]; Gacy and McMurray, Biochemistry 33, 11951 [1994]; and McMurray *et al.*, Biochemistry 33, 11960 [1994]). Within the enkephalin enhancer, mutations, which stabilize or destabilize a cruciform structure, resulted in increased or decreased transcription, respectively, without affecting the transcription factor binding (Comb *et al.*, EMBO J. 7, 3793 [1988]).

In the present invention, hairpin oligonucleotides, containing a duplex portion with a CRE, were introduced into cells and successfully functioned as decoys to alter gene expression.

In yet other embodiments, two hairpin forming synthetic oligonucleotides, each containing one of the sense- and antisense-*cis*-elements, respectively, and complementary to the other, in combination form a cruciform DNA. Such cruciform DNA can increase the potency of the transcription factor decoy to inhibit gene transcription. Similar DNA structures are known to be generated during genetic recombination (*See e.g.*, Holliday, Genet. Res. 5, 282 [1964]; Dressler and Potter, Annu. Rev. Biochem. 51, 727 [1982]; Gellert *et al.*, Cold Spring Harbor Symp. Quant. Biol. 43, 35 [1978]; and Panayotatos and Wells, Nature 289, 466 [1981]) and from palindromic sequences under the effect of supercoiling (*See e.g.*, Panayotatos and Fontaine, J. Biol. Chem. 262, 11364 [1987]; and Horwitz and Loeb, Science 241, 703 [1988]), indicating a biological role for such structures.

Figure 1 presents a model for cruciform decoys. This model is supported by work conducted on the enkephalin enhancer (Gacy and McMurray, Biochemistry 33, 11951 [1994]; and McMurray *et al.*, Biochemistry 33, 11960 [1994]). The hairpin decoys exert strong binding affinity for the transcription factors as shown in Figure 1(II), whereas duplex decoys are weak binding decoys (Figure 1(B)). The linear duplex exhibits poor binding for CREB and CREB preferentially bound and stabilized the hairpin form of the enhancer (Gacy and McMurray; and McMurray *et al.*, *supra*). While the present invention is not limited to any particular mechanisms, the transcription factor binding may stabilize the hairpin decoys and facilitate further binding of additional factors by inducing conformational changes as shown in Figure 1 (III and IV). The acidic pH facilitates and stabilizes the hairpin structure, and protein binding stabilizes the hairpin state due to its negative charge and/or by phosphorylation that increases the net negative charge around the enhancer (McMurray *et al.*, Proc. Natl. Acad. Sci. 88, 666 [1991]). The probability of duplex decoy (Figure 1(A)) formation from the hairpin decoy (Figure 1(I)) is small even in the absence of a stabilizing protein. It is shown that the hairpin state, once formed, shows a persistent stability even under conditions where the free energy difference between cruciform and duplex states favors the duplex (Gacy and McMurray; and McMurray *et al.*, *supra*). The probability of hairpin decoy (Figure 1(I)) formation from duplex decoy (Figure 1(A)) is small because the activation energy for the forward reaction, the hairpin formation from a linear duplex, is higher than that for the reverse reaction at neutral pH (Gacy and McMurray; and McMurray *et al.*, *supra*). The spontaneous interconversion between the protein-bound duplex state (Figure 1(B)) and protein bound cruciform (Figure 1(II)) is unlikely.

Because the probability of hairpin formation from a stable duplex of cellular *cis*-transcription element is small at neutral pH, and because the hairpin oligonucleotide may have a higher affinity for the transcription factor than the linear DNA, the exogenously supplied hairpin decoy oligonucleotide would be a more efficient competitor for the binding of cellular transcription factors than a linear duplex decoy, and thereby can interfere with transcription more efficiently *in vivo*.

The present invention contemplates all oligonucleotide structures that contain a CRE or similar sequence. These structures include, but are not limited to, linear duplex, hairpin, stem-loop, cruciform, bent, and any other secondary, tertiary, or quaternary structures.

In one embodiment of the present invention, a 24-mer CRE perfect palindrome oligonucleotide that is capable of forming a hairpin, 5'-TGACGTCATGACGTCATGACGTCA-3' (SEQ ID NO:2), or a non-palindromic hairpin forming oligonucleotide, 5'-GCTGACGTCGGCCTGACGTCAGC-3' (SEQ ID NO:3), penetrated into treated cells and competed with the cellular *cis*-element for the binding of sequence-specific CRE DNA-binding proteins, such as the 43 kDa CREB. The palindromic or hairpin-forming CRE oligonucleotide interfered with CRE-directed transcription in intact cells as determined by a transient transcription assay. The 24-mer CRE palindrome oligonucleotide produced potent growth inhibition in a variety of cancer cells including breast, prostate, lung, ovarian, colon, and epidermoid carcinomas, and multidrug-resistant (MDR) cancer cell lines of MCF7-TH (MDR-breast cancer) and HCT-15 (MDR-colon carcinoma). The growth of normal human mammary epithelial, lung epithelial, and human newborn foreskin fibroblast (*i.e.*, HS68 cells) cell lines was not affected by the CRE oligonucleotide. Treatment of nude mice bearing HCT-15 MDR colon carcinoma with 24-mer CRE oligonucleotide resulted in a potent inhibition of tumor growth. The CRE-oligonucleotide-induced growth inhibition accompanied changes in cell morphology and the appearance of apoptotic nuclei.

In other embodiments, the hairpin-forming oligonucleotide SEQ ID NO:10 demonstrated a strong inhibition of CRE-directed transcription in intact cells and showed very strong growth inhibition (*i.e.*, 70-80%). SEQ ID NO:11, the complement of SEQ ID NO:10, gave weaker growth inhibition (*i.e.*, 30%). Treatment with a combination of SEQ ID NO:10 and SEQ ID NO:11 gave over 80% growth inhibition. These oligonucleotides have a CRE sequence that differs from the CRE consensus sequence (*i.e.*, SEQ ID NO:1). These data demonstrate that hairpin forming oligonucleotides act as strong decoys, that sequences that

deviate from the CRE consensus act as strong decoys, and that a range of decoy potencies can be achieved for tailoring the desired competitive impact.

To demonstrate that the nucleotide sequence, and not the secondary structure alone, was required for decoy function, cells were treated with a nonsense sequence palindromic oligonucleotide (*i.e.*, an oligonucleotide comprising a perfect palindrome that forms a hairpin structure, but with no CRE sequence similarity). Such nonsense structural control oligonucleotides did not function as CRE decoys (*i.e.*, did not compete with native CRE sequences for binding to transcription factors).

Although the present invention is not limited to any particular mechanism, other control experiments suggested that the decoys bind to the transcription factor DNA-binding domain. First, undifferentiated F9 teratocarcinoma cells, a cell line that is unresponsive to cAMP, were treated with CRE decoys and no growth inhibition was observed. These results suggest that the decoys may act as growth inhibitors, at least in part, through binding to CREB since the CRE is nonfunctional in F9 cells although CREB is present (Gonzalez and Montminy, Cell 59, 675 [1989]). Second, KCREB, a CREB mutant that contains a mutation of a single amino acid in the DNA-binding domain, is known not to bind to native CRE sequences (Walton *et al.*, Mol. Endocrinol. 6, 647 [1992]). Cancer cells harboring KCREB exhibited decreased cell growth as compared to parental cells, and showed little or no response to the CRE decoy oligonucleotide treatment, indicating that the DNA binding domain was the region of interaction between the decoys and CREB and CREB-like transcription factors.

Furthermore, CRE-decoy oligonucleotides of the present invention were shown to penetrate cells and compete with the cellular CRE-elements for binding transcription factor complexes containing CREB. Nuclear extracts from cells treated with the 24-mer CRE oligonucleotide (150 nM, for 2 days) demonstrated a marked decrease in formation of the CRE-protein complex in the mobility shift assay as compared to control (saline-treated) cells as show in Figure 12A (lanes 4, 8, and 12). In this Figure, "C" represents saline-treated cells; "CRE" represents 24-mer-CRE palindromic oligonucleotide treated cells (150 nM, for 2 days); and "CREC" represents 24-mer CRE-mismatch control oligonucleotide-treated cells. A CREB antibody caused supershift, indicating the presence of CREB protein within the labeled protein-DNA complexes (lanes 2, 6, and 10). The two-base mismatched control oligonucleotide treatment did not affect the CRE-protein complex formation (lanes 3, 7, and 11). A Western blot analysis for CREB protein in untreated, CRE oligo treated, and control

oligo treated cells demonstrated no change in CREB protein level as shown in Figure 12B, indicating that the CRE-decoy treatment did not affect CREB levels in the cells. These results were demonstrated in MCF7 (breast carcinoma), MCF-10A (normal human mammary epithelial cells), and LNCaP (prostate carcinoma) cells. The above results further demonstrate that the CRE-decoy oligonucleotides of the present invention successfully competed with the cellular CRE-enhancer for binding of sequence-specific DNA-binding proteins.

The data show that the CRE-transcription factor decoy can modulate *in vivo* gene transcription and restrain tumor growth *in vivo*. Thus, this technology offers great promise as a tool for treating diseased conditions and can also be used for defining cellular regulatory processes.

The specificity of the growth inhibitory effect of the decoy oligonucleotides against CRE-transcription factors on cancer cells is supported by several lines of evidence; (i) multiple different CRE decoy oligonucleotides produced potent growth inhibition of cancer cells but not normal cells, *in vitro and in vivo*, whereas, mismatched control oligonucleotides (*i.e.*, oligonucleotides similar to the decoys but containing mismatched nucleotide pairs, such that self-hybridization does not occur--non-duplex oligonucleotides) did not inhibit growth; (ii) the administration of CRE decoy oligonucleotides, but not mismatched oligonucleotides, markedly inhibited CRE DNA-protein complex formation and CRE-directed transcription activity in both cancer cells and normal cells; (iii) cellular uptake of decoy oligonucleotides and mismatched oligonucleotides was similar for cancer cells and normal cells; and (iv) the specific growth inhibitory effect toward cancer cells correlated with induction of cell differentiation/apoptosis.

i) Inhibition of cAMP-dependent protein kinase (PKA) and phosphoenol pyruvate carboxykinase (PEPCK)

Experiments conducted during the development of the present invention demonstrate that the methods and compositions of the present invention block both basal and cAMP-induced expression of CRE-containing genes. A group of cAMP-responsive genes, such as somatostatin and phosphoenol pyruvate caroxykinase (PEPCK) contain the CRE which lies within the first 150 bp of the 5'-flanking region of the gene (Roesler *et al.*, *supra*). Therefore, these elements could be regarded as basal enhancers, in addition to functioning as inducible enhancers (Maniatis *et al.*, *Science* 236: 1237-1245 [1987]). A role for the CRE as a basal transcription element was suggested in deletion analysis of the PEPCK promoter-

regulatory region (Short *et al., J. Biol. Chem.* 261: 9721-9726 [1986]). When the CRE was deleted from the promoter, the basal level of gene transcription was reduced and the responsiveness of the promoter to cAMP in hepatoma cells was abolished. CRE binding protein binds to the CRE in a cAMP-independent manner (Roesler *et al., supra*; Montminy *et al., Nature* 328: 175-178 [1987]). This binding may stimulate basal transcription by interacting with proximal promoter element, such as the TATA box binding factor and/or RNA polymerase II. The cAMP-induced phosphorylation of CREB could lead to a higher-order complex formation with the basic transcription factor. Thus, the CRE can function as both a basal and an inducible transcription element.

Treatment with the CRE-oligonucleotides of the present invention interfered with both basal and cAMP-induced transcription of an exogenously supplied CRE-containing gene as shown in Figure 8. In this Figure, the effect of CRE-oligonucleotides on the transactivation of somatostatin-CAT fusion gene are shown (Δ-71-CAT; Montminy *et al.*, Proc. Natl. Acad. Sci. 83:6682 [1986]). Transfection and CAT-activity assays were performed using standard procedures and are described below. "C" represents saline-treated control cells; "CRE" represents CRE-oligonucleotide-treated cells (150 nM, 2 days); and "CREC" represents control nonsense-sequence palindromic oligonucleotide-treated cells (150 nM, 2 days). Standard deviation for each CAT assay was less than 10%, and results are representative of 2-4 independent transfections.

Furthermore, the CRE palindrome oligonucleotide treatment brought about a marked reduction in the mRNA levels of the catalytic (Cα) and regulatory (RIα) subunits of cAMP-dependent protein kinase (PKA) and PEPCK in MCF7 breast cancer cells as shown in Figure 9. Total cellular RNA (from 2 x 10^6 cells) preparation and Northern blotting were performed using standard procedures and are described below. ^{32}P-labeled probers were: the 1.5-kb cDNA clone containing the entire coding region of human RIα (kindly provided by Tore Jahnsen); the 1.1-kb full-length sequence of the human Cα (kindly provided by Steven K. Hanks); the 0.9-kb clone containing the human PEPCK (ATCC); and a 29-mer oligonucleotide probe for human 28S rRNA (Clontech). "C" represents saline-treated control cells; "CRE" represents CRE-oligonucleotide-treated cells (150 nM, 2 days); and "CREC" represents control nonsense-sequence palindromic oligonucleotide-treated cells (150 nM, 2 days). The data represent one of 3-4 independent experiments that gave similar results.

In non-cancerous MCF-10A cells, the reduction in the mRNA levels was small, although the expression of these genes, particularly of RIα and Cα genes was very low. In

contrast, the control oligonucleotide had no effect on the mRNA levels of the RIα, Cα and PEPCK (Figure 9).

PEPCK and the PKA R and C subunit genes are cAMP-inducible genes (Roesler *et al.*, *supra*; Tasken *et al.*, *Mol. Endocrinol.* 5: 21-29 [1991]). The promoter region of the porcine RIα gene contains a CRE consensus sequence as well as the AP-2 recognition site (Nowak *et al.*, *Eur. J. Biochem.* 167: 27-33 [1987]). Transcription factor binding to both of these sequences is believed to be involved in cAMP regulation of gene transcription (Roesler *et al.*, *supra*). The promoters of PKA R and C subunit genes are TATA-less and GC rich, and multiple transcription initiation sites were identified within the GC rich region (Nowak *et al.*, *supra*; Chrivia *et al.*, *J. Biol. Chem.* 263: 5739-5744 [1988]). Such GC rich regions have been associated with transcription initiation sites of many constitutively expressed housekeeping genes (Bird, *Nature* 321: 209-213 [1986]). The observation that the CRE-oligonucleotides of the present invention inhibit the basal expression of RI and C genes (Figure 9) suggests that the CRE-oligonucleotide can compete with the *cis*-CRE element in binding CREB, although an understanding of the mechanism is not necessary to practice the present invention, and it is not intended that the present invention be so limited. Because the CRE-oligonucleotide can interfere with CREB binding to the cis-element, it is expected that the oligonucleotide could produce a more profound effect on the mRNA reduction under cAMP-induced conditions. Thus, the CRE-oligonucleotide can interfere with both basal and cAMP-induced expression of the endogenous CRE-containing genes.

ii) Inhibition of AP-1 binding

Experiments conducted during the development of the present invention demonstrate that the methods and compositions of the present invention interfere with the transcriptional regulation caused by a variety of signaling pathways. For example, CREB is known to associate with a variety of other transcription factors (*e.g.*, members of the *jun/fos* family) (Habener, *Mol. Endocrinol.* 4: 1087-1097 [1990]). The products of the proto-oncogenes *jun* and *fos* bind as a heterodimeric complex to a DNA sequence element TRE (AP-1) binding site (Rauscher *et al.*, *Cell* 52: 471-480 [1988]), whereas CREB-1 homodimer and CREB-2/ATF heterodimer bind the CRE sequence (Habener, *supra*). However, jun/jun homodimer binds to both CRE and TRE (Nowak *et al.*, *supra*), CREB-2 (ATF-2)/jun heretodimer binds CRE (Habener, *supra*; Ellis *et al.*, *J. Molecular Endocrinology* 14: 191-198 [1995]), and c-fos

is cAMP-inducible (Roesler *et al.*, *supra*). These results demonstrate that AP-1 and CRE are interrelated.

The effect of CRE-oligonucleotide treatment on AP-1 binding was examined as shown in Figure 10. In this Figure, binding site specificity was tested by EMSA with the ^{32}P-labeled oligonucleotide [double-stranded AP-1, SP-2, and Oct-1 (Promega)] in the absence and presence of unlabeled competitor, as indicated. Nuclear extracts were prepared from cells treated with saline (C), CRE-oligonucleotide (CRE) or control mismatched oligonucleotide (CREC) at 150 nM for 2 days, and the EMSA was performed using standard procedure and described below. Data represent one of three independent experiments that gave similar results.

The nuclear extracts from cells treated with CRE-palindromic oligonucleotide demonstrated a marked reduction in formation of the AP-1 DNA-protein complex in the mobility shift assay compared to control cells (Figure 10, lane 4). Two-base mismatched control oligonucleotide treatment had no effect on the AP-1 DNA-protein complex formation (Figure 10, lane 3). By comparison, a CRE-decoy oligonucleotide had no effect on Sp-1 or Oct-1 DNA-protein binding (Figure 10, lanes 8 and 12).

Additionally, experiments were conducted to determine the effect of CRE-decoy oligonucleotide treatment on the expression of *c-fos* gene, which is known to be cAMP responsive (Roesler *et al.*, *supra*). As shown in Figure 13, the CRE-decoy brought about a marked decrease in the TPA-inducible mRNA level of *c-fos*. At the indicated times (Figure 13), cells were harvested. RNA preparation and Northern blot analysis were performed as described in the Example section. The control mismatched oligonucleotide had no effect on the *c-fos* expression. A cAMP unresponsive gene, such as PKC-α was not affected by the CRE-oligonucleotide treatment.

As described in the Examples provided below, a variety of methods find use to deliver the decoys of the present invention into target cells. Methods for *in vivo* and/or *in vitro* delivery include, but are not limited to, oral intake, injection (*e.g.*, subcutaneous, intraperitoneal, intravenous, intramuscular, or other injection methods), direct exposure in aqueous or media solution, transfection (*e.g.*, calcium phosphate, electroporation, DEAE-dextran based, and lipid mediated), transgenic expression (*e.g.*, a decoy expression system delivered by microinjection, embryonic stem cell generation, or retroviral transfer), time-

release pellet, micropump adminstration, or any of the other commonly used nucleic acid delivery systems known in the art.

A sufficient concentration of decoys are added to target cells to guarantee significant competition with native enhancer sequences. For example, it has been estimated that when 10 μM of phosphorothioate modified oligonucleotide are incubated with 2.5 X 10^6 cells/ml, there are 10^7 to 10^8 molecules of the oligonucleotide per cell (Bielinska *et al.*, Science 250, 997 [1990]). If only a small fraction of these penetrated into the cell nuclei (*e.g.*, 10%), each cell would contain over a million copies of the oligonucleotide. This is in 100 to 1000-fold excess over the likely number of transcription factors present in a given cell (Lenardo *et al.*, Proc. Natl. Acad. Sci. 85, 8825 [1988]).

For example, during the development of the present invention, two administrative routes were evaluated in the examination of antitumor effects of the CRE-palindromic oligonucleotides on MCF-7 cells implanted in mouse mammary glands. Intraperitoneal injection (0.1 mg/mouse, daily, 5x/week for 4 weeks) and interscapular implantation of a time-release pellet (3 mg/60 days release/mouse [Innovative Research of America]) were used. In both modalities, the CRE-palindromic oligonucleotide caused significant inhibition in tumor growth as compared to the saline-treated or the scrambled control oligonucleotide-treated tumors without systemic toxicity. Treatment with the pellet implantation was more effective than i.p. injection on tumor growth inhibition. Although an understanding of the mechanism is not necessary to practice the present invention, and it is not intended that the present invention be so limited, this may be because the pellet constantly released the oligonucleotide throughout the experimental time period and maintained constant plasma concentration of oligonucleotide. As compared to a control tamoxifen pellet (5 mg/60days release), the CRE oligonucleotide-pellet (3 mg/60 days release) produced a 3-fold greater potency in growth inhibition. This illustrates that a variety of administrative routes find use with the present invention, and that the present invention provides a powerful new antineoplastic agent.

In addition to providing an effective means of controlling cancer cell proliferation, the CRE decoy oligonucleotides of the present invention find use in many other applications. A large number of genes are transcriptionally regulated through cAMP response elements. Many of these gene encode for regulatory molecules that control the expression of other genes. For example, CREB interacts with CBP which is involved in the transcriptional activation of p53 (Gu *et al.*, Nature 387, 819 [1997]). In addition, CREB is known to

associate with (*e.g.*, heterodimerize) a variety of other transcription factors (*e.g.*, members of the Jun/Fos family, Nilsson *et al.*, Cell Growth and Differentiation 8, 913 [1997]; and CBP, which can be competitively bound away from other transcription factors that it co-activates such as AP-1 and RARs, (Agadir *et al.*, Cancer Research 57, 3444 [1997]).

One of many possible examples for further use of CRE decoys is in the regulation of hepatitis B virus metabolism. The X gene product (*i.e.*, pX) of the hepatitis B virus is an important transactivator of a variety of viral and cellular genes (Williams and Andrisani, Proc. Natl. Acad. Sci. 92, 3819 [1995]). pX has been shown to interact with transcription factors that bind to CREs such as CREB and ATF. The use of CRE decoys may help prevent pX action and thus interfere with the virus life cycle. CRE-like sequences have also been identified in the promoters of other viruses (*e.g.*, HTLV-1).

Surprisingly, the decoys of the present invention were also found to inhibit protein kinase A (PKA) activity in decoy-treated cancer cells but not normal cells. Cultured ovarian cancer cells were treated with and without CRE decoy oligonucleotide using the methods of Example 1 below. Extracts were analyzed by column chromatography for PKA activity. Untreated cancer cells demonstrated two peaks corresponding to Type-1 and Type-2 PKA, while CRE decoy-treated cells exhibited no peaks (*i.e.*, PKA activity was wiped out). HS68 cells (*i.e.*, non-cancerous cells) did not show any differences in PKA activity between treated and untreated samples. These data demonstrate that CRE-decoy oligonucleotides inhibit PKA activity in cancer cells but not in normal cells.

Additionally, the CRE-decoy oligonucleotides of the present invention were found to regulate the p53 signalling pathway. It was discovered that CRE-decoy oligonucleotide treatment of MCF7 cells, which had a lower level of wild type p53, resulted in marked increase of p53 protein. Pulse chase experiments demonstrated that the marked increase of p53 was related to increased stability of the protein. In transient transcription assays, it was found that decoy oligonucleotide treatment induced p53-luciferase activity. p21Cip1/WAF1 protein was also induced by the CRE-decoy oligonucleotide treatment and the p21 induction accompanied a reduction in cdk-2-dependent kinase and cyclin E-dependent kinase activities, and pRB dephosphorylation. Thus, the stabilization and activation of p53 may contribute to the growth inhibition induced by CRE-transcription factor decoy oligonucleotide in MCF7 breast cancer cells, although an understanding of the mechanism is not necessary to practice the present invention, and it is not intended that the present invention be so limited.

Thus, by targeting the desired cells with appropriate exposure (*i.e.*, time, concentration, and affinity) of decoy, the present invention provides a means of regulating many physiological and cellular processes that are mediated by cAMP.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be read as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); IM (intramuscular); IP (intraperitoneal); IV (intravenous or intravascular); SC (subcutaneous); H_2O (water); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); nM (nanomolar); U (units); V (volts); MW (molecular weight); µCi (microcurrie); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); ab (antibody); IC_{50} (50% inhibitory concentration); CRE (cAMP response element); CREB (cAMP response element-binding protein); DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate); SS-CAT (somatostatin-chloramphenicol acetyltransferase); CAT (chloramphenicol acetyltransferase); TLC (thin layer chromatography); TPA (12-O-tetradecanoylphorbol 13-acetate); DTT (dithiothreitol); HCl (hydrochloric acid); $MgCl_2$ (magnesium chloride); KCl (potassium chloride); NaCl (sodium chloride); OD_{280} (optical density at 280 nm); OD_{600} (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PCR (polymerase chain reaction); PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); RT-PCR (reverse transcription PCR); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); EDTA (Ethylenediaminetetraacetic Acid); w/v (weight to volume); v/v (volume to volume); MDR (multidrug-resistant); MCF7 (human breast adenocarcinoma cells); MCF10A (human mammary gland cells); LNCaP (human metastatic prostate adenocarcinoma); A549 (human lung carcinoma cells); OVCAR 8 (human ovarian carcinoma cells); LS 174T (human colon adenocarcinoma cells); KB (human epidermoid carcinoma cells); HCT-15 (MDR human colon adenocarcinoma cells); MCF7-TH (MDR-breast cancer cells); L-132 (human embryonic lung

cells); EMBA (Electrophoretic mobility shift assay); Bio-Rad (BioRad, Richmond, CA); and Sigma (Sigma Chemical Co., St. Louis, MO).

Oligonucleotides comprising the following sequences were used in the Examples below.

24-mer CRE-palindrome (SEQ ID NO:2) : 5'-TGACGTCA TGACGTCA TGACGTCA-3'

24-mer CRE-palindrome control (SEQ ID NO:8) : 5'-TGTGGTCA TGTGGTCA TGTGGTCA-3'

23-mer CRE-hairpin (SEQ ID NO:3) : 5'-GCTGACGTCGGCCTGACGTCAGC-3'

23-mer CRE-hairpin control (SEQ ID NO:9) : 5'-GCTGACCACGCCGTGTGGTCAGC-3'

24-mer CRE-hairpin sense (SEQ ID NO:10) : 5'-TGCCGTCATGCCGTCATGCCGTCA-3'

24-mer CRE-hairpin antisense (SEQ ID NO:11) : 5'-TGACGGCATGACGGCATGACGGCA-3'

EXAMPLE 1

CRE-decoy oligonucleotide inhibited CRE DNA-protein complex formation

A 24-mer single strand oligonucleotides comprising a CRE palindrome (*i.e.*, multiple copies of a CRE consensus sequence) were introduced into a variety of culture cells. The oligonucleotide, 5'-TGACGTCATGACGTCATGACGTCA-3' (SEQ ID NO: 2), can contain unmodified phosphodiester bonds, or can contain phosphorothiorate, phosphoramidite, methylphosphonate derivatives, or other modifications to help provide stability and facilitate entry into the cells. Because the CRE oligonucleotide is palindromic, it can self-hybridize to form a duplex and provide a binding site for transcription factors that interact with CREs,

including, but not limited to, the 43 kDa CREB (CRE binding protein) (Montminy and Bilezikjian, Nature 328, 175 [1987]).

Cells were treated with saline (*i.e.*, control cells), the CRE 24-mer (150 nM for 2 days), or a control oligonucleotide and nuclear extracts were prepared using the method of Dignam (Dignam *et al.*, Nucleic Acid Res. 11, 1475 [1983]). The control oligonucleotide was 5'-TGTGGTCATGTGGTCATGTGGTCA-3' (SEQ ID NO: 8), which is a copy of the CRE 24-mer with two mismatched bases included in each consensus sequence motif (*i.e.*, it will not bind as well to transcription factors as will the consensus sequence oligonucleotide). Nuclear extracts were obtained from the cells and were analyzed by a mobility shift assay to compare the amount of CRE-protein bound complex between treated, untreated, and control samples. The DNA binding assay was performed by a method modified from that of Fried and Crothers (Fried and Crothers, Nucleic Acid Res. 9, 6506 [1981]). Briefly, nuclear extracts (10 μg protein) were pre-incubated with poly(dI-dC) • poly (dI-dC) (1 μg), DTT (0.3 mM), and binding buffer (12 mM Tris pH 7.9, 2 mM $MgCl_2$, 60 mM KCl, 0.12 mM EDTA, and 12% glycerol) with or without antiserum (2-4 μl) for 30 min at 4 °C. ^{32}P-labeled oligonucleotide (double-stranded CRE triplet, 5'-CCTGACGTCATGACGTCATGACGTCA-3'; SEQ ID NO:12) was then added and the reaction mixtures were incubated for 10 min at 37 °C. The reaction mixtures were then separated on a 4% polyacrylamide gel at 200 V for 1.5 hours. The gel was dried and autoradiographed. AbCREB, a CREB antibody, was added to determine the presence of CREB protein within the labeled protein-DNA complexes.

As shown in Figure 2, the nuclear extracts from cells treated with the 24-mer CRE oligonucleotide demonstrated a marked decrease in formation of the CRE-protein complex in the mobility shift assay compared to untreated cells. In this figure, saline treated cells are represented by "C," CRE 24-mer treated cells by "CRE," and control 24-mer treated cells by "CREC." Samples that were treated with AbCREB antibody are designated with a "+." The two arrows designate the locations of the CRE-protein complex on the gel. As is clear from the Figure 2, the nuclear extracts from cells treated with the two-base mismatched control oligonucleotide showed the same or similar intensity band of CRE-protein complex as that demonstrated by the nuclear extracts of saline treated cells. These results were demonstrated in MCF7 (breast cancer), MCF10A (normal human mammary epithelial cell), and LNCaP (prostate cancer) cell lines. Furthermore, as shown in Figure 10, CRE-protein formation was not inhibited by nonsense sequence palindrome oligonucleotide containing no CRE, or Oct-1, AP-1 and SP-1 sequences.

This Example provides a screening method to assay oligonucleotide candidates for their decoy capabilities and efficiencies. A candidate can be tested in place of the 24-mer (SEQ ID NO: 2) as described above. The percentage inhibition of transcription factor binding to the ^{32}P-labeled CRE oligonucleotide (double-stranded CRE triplet, 5'-CCTGACGTCATGACGTCATGACGTCA-3'; SEQ ID NO:12) can be calculated and decoy efficiency thus determined. For determination of binding competition to transcription factors other than CREB, other antibodies can be used (*e.g.*, antibodies raised against cyclic AMP-responsive element modulators (CREMs), ATF-1, Tax, pX, C/EBPs, Jun, Fos, CBP, or other desired transcription factors or proteins).

EXAMPLE 2

CRE-decoy oligonucleotide interfered with CRE-directed transcription

The CRE oligonucleotide successfully interfered with the CRE-directed transcription in intact cells. To analyze the effect of CRE oligonucleotide in CRE-directed transcription, a transient transcription assay was performed using a reporter plasmid, somatostatin Δ-71 (CRE-containing promoter)-CAT (chloramphenicol acetyltransferase) fusion gene (Montminy *et al.*, Proc. Natl. Acad. Sci. 83, 6682 [1986]). MCF7 cells were transfected with 5 μg of somatostatin-chloramphenicol acetyltransferase fusion gene (Δ-71 SS-CAT plasmid) and 4 μg of CRE (*i.e.*, the 24-mer described in Example 1) or control oligonucleotide by the use of transfection reagent N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate (DOTAP). After 5 hours, fresh medium was added, and the cells were harvested at 48 to 72 hours, then assayed for CAT activity. Some samples were also treated with forskolin (10 μM), an compound with adenyl cyclase activating properties (*i.e.*, a compound that increases intracellular cAMP concentrations), for the final 24 hours. Cell lysates were prepared as described by Gorman (Gorman, High efficiency gene transfer into mammalian cells, *DNA Cloning*, Vol II, pp 143-165, IRL press, Oxford, England [1985]). Lysates (75 μg of protein) were incubated with 0.4 μCi of [^{14}C]chloramphenicol, 0.53 mM acetyl-CoA, and 250 mM Tris-HCl, pH 7.8 for 90 minutes at 37 °C. Under these conditions, CAT activity was linear with time. Reaction products were analyzed by thin-layer chromatography (Gorman, *supra*).

As shown in Figure 3, transfection of cells with Δ-71 CAT plus the single stranded CRE palindrome oligonucleotide resulted in a greater than 90% inhibition of the CRE-directed transcription compared to cells transfected with Δ-71 CAT alone. In this Figure, C represents

control cells treated with saline and the Δ-71 CAT alone and CRE-P represents cells treated with the CRE palindrome oligonucleotide and Δ-71 CAT. The lane labeled CRE-HP-S represents cells treated with Δ-71 CAT and single stranded oligonucleotide that forms a hairpin loop, 5'-TGCCGTCATGCCGTCATGCCGTCA-3' (SEQ ID NO:10), where the double-stranded stem forms a CRE binding site (*i.e.*, CRE transcription factors should bind to it). Lanes labeled with a "+" had forskolin added to them for the final 24 hours. Addition of the two–base mismatched control oligonucleotide (*i.e.*, the control oligonucleotide described in Example 1), which does not self-hybridize to form a duplex, had no inhibitory effect on the CAT activity. Addition of the nonsense-sequence palindromic oligonucleotide that contained no CRE sequence had no inhibitory effect on the CAT activity. None of the oligonucleotides inhibited the Simian virus 40 (SV40) enhancer, which contained no recognizable CRE enhancer element. However, the 24-mer CRE-hairpin sense oligonucleotide strongly inhibited CAT activity. These results were also demonstrated in MCF10A and LNCaP cells.

Furthermore, untreated MCF-7 cells exhibited a relatively high level of basal somatostatin-CAT activity, and forskolin treatment had only a small stimulatory effect (Figure 8). In contrast, LNCaP prostate cancer cells and non-cancerous MCF-10A cells exhibited very low levels of basal CAT activity, and forskolin greatly stimulated CAT activity (5-10 fold). This forskolin-stimulated CAT activity was also almost completely abolished by the CRE-oligonucleotide treatment. The disparity in the basal CAT activity observed in different cells may reflect varying degrees of cAMP-dependent protein kinase activation and CREB-phosphorylation in the cell, although an understanding of the mechanism is not necessary to practice the present invention, and it is not intended the the present invention be so limited. Further, the CRE-oligonucleotide was capable of inhibiting both basal and cAMP-stimulated CAT activities.

EXAMPLE 3

Cellular uptake of CRE-decoy oligonucleotide

To examine the efficacy of cellular incorporation of the CRE oligonucleotides, we incubated ^{32}P-labeled samples of CRE-palindrome oligonucleotide and control oligonucleotide with MCF7 and MCF10A cells. Cell-associated radioactivity was quantified. Within 5 hrs, about 10% of the total input oligonucleotide accumulated in the cell and the incorporation

continued to rise thereafter reaching 20-25% maximum levels at 24 hr of oligonucleotide incubation. The amounts and the rates of the incorporation of oligonucleotide were similar between MCF7 and MCF10A cells and between the CRE-decoy oligonucleotide and control oligonucleotide.

EXAMPLE 4

CRE-decoy oligonucleotide inhibited cancer cell growth *in vitro*

The 24-mer CRE palindrome oligonucleotide produced potent growth inhibition in a variety of cancer cells including MCF7 (breast cancer), LNCaP (prostate cancer), A549 (lung carcinoma), OVCAR 8 (ovarian carcinoma), LS174T (colon carcinoma), SW480 (colon carcinoma), KB (epidermoid carcinoma), and multidrug-resistant (MDR) cancer cell lines of MCF7-TH (MDR-breast cancer) and HCT-15 (MDR-colon carcinoma). Importantly, the growth of normal cells, MCF10A (human mammary epithelial cell), Hs68 (human newborn foreskin fibroblast), NIH/3T3 fibroblasts, and L-132 (human lung epithelial cell) was not affected by the CRE oligonucleotide.

Cells (0.25 - 1 x 10^5/well) were plated in a 6-well plate containing the growth medium at 37°C. The 24-mer-CRE palindrome oligonucleotide (*i.e.*, as described in Example 1) or two-base mismatched control oligonucleotide (*i.e.*, as described in Example 1) was added, one day after seeding, to duplicate wells at varying concentrations (50-200 nm). To increase the delivery of oligonucleotides into the cell, transfection reagent DOTAP was added along with the oligonucleotides. At 5 hours of incubation, the medium was removed, and fresh medium without oligonucleotide and DOTAP was added. Saline treated (untreated), DOTAP treated (0 concentration), CRE, or CRE control oligonucleotide treated cells were harvested after 4 days of treatment, and cell numbers were counted in duplicate by a Coulter Counter. Data, as shown in Figure 3, represent mean ± S.D. obtained from 3 separate experiments. In this figure, lines with closed circles represent cells treated with the 24-mer-CRE palindrome oligonucleotide, while lines with open boxes represent cells treated with the control, mismatch oligonucleotide.

As shown in Figure 4, the growth inhibition of cancer cells was achieved at nanomolar concentrations of CRE oligonucleotide (IC_{50}, 100-150 nM). As shown, growth was inhibited in each of the cancer cell lines, but was unaffected in the normal cells (*i.e.*, MCF-10A and L-132 cells). The growth inhibition was CRE-sequence specific as the two-base mismatched

control oligonucleotide had no growth inhibitory effect. In separate experiments, the 24-mer CRE-hairpin sense and antisense oligonucleotides at 150 nM concentration demonstrated 70% and 30% growth inhibition, respectively, and in combination produced over 80% growth inhibition.

EXAMPLE 5

CRE-decoy oligonucleotide inhibited tumor growth *in vivo*

HCT-15 human MDR colon carcinoma tumor cells (2 x 10^6 cells) were inoculated subcutaneously into the left flank of athymic mice. The CRE or control oligonucleotide was injected intraperitoneally into mice at 0.01 – 0.1 mg/0.1 ml saline/mouse, daily, 5 x/week for 4 weeks, when tumor size reached 30–50 mg ~10 days after cell inoculation. Tumor volumes were obtained from daily measurements of the longest and shortest diameters and calculated by the formula, $4/3\pi r^3$ where r = (length + width)/4.

As shown in Figure 5, treatment of nude mice bearing HCT-15 human MDR colon carcinoma with 24-mer CRE oligonucleotide (open boxes = 0.01 mg; closed boxes = 0.1 mg) resulted in greater than 85% inhibition of tumor growth as compared to the saline treated control tumors (open circles), without causing toxicity. Two-base mismatched control oligonucleotide (closed circles) had no growth inhibitory effect. Data represent means ± S.D. of 5-7 tumors in each group.

EXAMPLE 6

CRE-oligonucleotide treatment induced differentiation/apoptosis

CRE-oligonucleotide treatment resulted in changes in cell morphology and appearance of apoptotic nuclei (OVCAR 8), as shown in Figure 6. The changes in cell morphology and apoptotic nuclei were not observed in the saline or control oligonucleotide treated cells. To examine whole cell morphology, OVCAR8 cells, untreated (Control) or treated with CRE-palindrome oligonucleotide (CRE Oligo; as described in Example 1) or control oligonucleotide (Control Oligo; as described in Example 1), were washed with PBS, fixed with 70% methanol for 5 min, and stained with Giemsa (Bio-Rad) for 15 min. Figure 6,

section A, shows whole cell morphology. After staining, the whole cells were visualized under an inverted microscope at x240. As shown, cells treated with the CRE decoy oligonucleotides exhibited the characteristics of apoptotic cells (*e.g.*, loss of cell junctions, loss of micovilli, condensed cytoplasm, margination of nuclear chromatin into discrete masses, compacting of mitochondria and ribosomes, dilation of the endoplasmic reticulum, and break-up of cells into several membrane bound bodies), while cells treated with the mismatch oligonucleotides appeared similar to the untreated cells. Figure 6, section B shows nuclear morphology. To assay nuclear morphology (*i.e.*, apoptotic nuclei), cells were washed with PBS, fixed with 70% ethanol for 1 hr, and stained with 1 mM Hoechst 33258 (Sigma) for 30 min (Oberhammer *et al.*, Proc. Natl. Acad. Sci. 89, 5408 [1992]). The nuclear morphology of cells was visualized by a fluorescence microscope (Olympus BH2) at x2600. As shown, nuclei from cells treated with the CRE decoy oligonucleotides exhibited the characteristics of apoptotic nuclei, while nuclei from cells treated with the mismatch oligonucleotides appeared similar to the untreated nuclei.

The bcl-2 protein promotes cell survival (Vaux *et al.*, Nature 335, 440 [1988]) by inhibiting the process of apoptosis (Haldar *et al.*, Arch. Biochem. Biophys. 315, 483 [1994]; and Miyashita and Reed, Blood 11, 151 [1993]). MCF7 cells have been shown to express an increased level of bcl-2 protein (Haldar *et al.*, Cancer Res. 54, 2095 [1994]), consistent with their cancerous phenotype.

Bcl-2 protein levels were measured by standard western blotting techniques known in the art using Anti-bcl-2 antibody. As shown in Figure 7, CRE-decoy oligonucleotide treatment markedly reduced bcl-2 level in MCF7 cells. Lanes marked "S" were from saline treated cells, "CRE" from CRE-palindrome oligonucleotide (150 nM, 4 days) treated cells, and "C" from CRE-control oligonucleotide treated cells. The normal mammary epithelial cell line, MCF-10A, contained a low level of bcl-2, and the CRE oligonucleotide treatment did not alter the bcl-2 level in these normal cells. The control oligonucleotide had no effect on bcl-2 protein level in any of the cell types.

EXAMPLE 7

Synthesis of CRE-decoy and control phosphorothioate oligonucleotides

CRE-decoy and control oligonucleotides used in the present invention (*i.e.*, 24-mer phosphorothioate oligonucleotides) were synthesized using β-cyanoethylphosphoramidite

ch mistry on automated DNA synthesizer (Pharmacia Oligo Pilot II). Deprotection and purification of the oligonucleotides followed standard procedures (Padmapriya *et al.*, Antisense Res. Dev. 4: 185-199 [1994]). Analysis of the purified oligonucleotide was carried out using capillary gel electrophoresis (CGE) and polyacrylamide gel electrophoresis (PAGE). The purity of the oligonucleotide based on CGE was 95% full length and 5% n-1, n-2 products.

EXAMPLE 8

Treatment of cells in culture with CRE-oligonucleotides

Cells (0.25 - 1 x 10^5 cells/well) were plated in 6-well plates containing the growth medium at 37 °C. To increase the delivery of oligonucleotide into the cell, oligonucleotides were treated with cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP). The CRE decoy and control oligonucleotides were added one day after seeding to the wells at varying concentrations (50-200 nM) in the presence of DOTAP. At 5 hours of incubation, the medium was removed, and fresh medium without oligonucleotide and DOTAP was added. Cells were harvested, and cell numbers were counted in duplicated by a Coulter Counter.

EXAMPLE 9

Production of stable transfectants

MCF7 cells (3 x 10^5 cells/60 mm dish) were transfected with 6 μg of KCREB plasmid, a dominant negative mutant form of CREB using DOTAP. Stably transfected cells were selected by growing cells in the presence of Geneticin (400 g/mL) (G418, Gibco-BRL). The G418 resistant colonies were isolated after three weeks of selection.

EXAMPLE 10

Electrophoretic mobility shift assay

Nuclear extracts were prepared by the method of Dignam *et al.* (Dignam *et al.*, Nucleic Acid Res. 11: 1475-1489). EMSA assay was performed by a method of Fried and Crothers (Fried *et al.*, Nucleic Acid Res. 9: 6506-6525 [1981]). Nuclear extracts (5 μg protein) were

preincubated with poly(dI-dC)•poly(dI-dC) (2μg), DTT (0.3 mM), and reaction buffer (12 mM Tris pH 7.9, 2 mM MgCl2, 60 mM KCl, 0.12 mM EDTA, and 12% glycerol) with or without CREB antiseum (1-2 μL) for 30 minutes at 4 °C. ^{32} P-labeled oligonucleotides (ds oligonucleotides with one copy of CRE; 5'-AGAGATTGCCTGACGTCAGAGAGTAG-3' (SEQ ID NO:15), Oct1; 5'-TGTCGAATGCAAATCACTAGAA-3' (SEQ ID NO:16), AP1; 5'-CGCTTGATGAGTCAGCCGGAA-3' (SEQ ID NO:17) and SP1; 5'-ATTCGATCGGGGCGGGGCGAGC-3' (SEQ ID NO:18), (Promega) were then added and the reaction mixtures were incubated for 10 minutes at 37 °C. The reaction mixtures were then separated on a 4% non-denaturing polyacrylamide gels at 200 V for 2 hours. The gel was dried and autoradiographed. AbCREB, CREB antibody (Santa Cruz Biotech.) was used for supershift.

EXAMPLE 11

Transient transcription assay of somatostatin-chloramphenicol acetyltransferase (SS-CAT)

Cells (5 x 10^5 cells/60 mm dish) were transfected with 3 μg of SS-CAT fusion gene plasmid and 4 μg of CRE or control oligonucleotide using DOTAP. After 24 hours, fresh medium was added, and the cells were harvested at 48-72 hours, then assayed for CAT activity. In other embodiments, cells were treated with forskolin (10 μM) for the final 24h. Cell lysates were prepared as described by Gorman (Gorman, DNA Cloning, vol. II, pp. 143-165, IRL Press, Oxford, England [1985]). Lysates (100 μg of protein) were incubated with 0.4 μCi of [^{14}C]chloramphenicol, 0.53 mM acetyl-CoA, and 250 mM Tris-HCl, pH 7.8 for 90 minutes at 37 °C. Under these conditions, CAT activity was linear with time. Reaction products were analyzed by thin layer chromatography (Gorman, *supra*). TLC plate was autoradiographed.

EXAMPLE 12

Stability tests of oligonucleotide

Cells (4 x 10^5 cells/60 mm dish) were incubated in growth medium containing 6 ng of ^{32}P-labeled CRE or palindromic control oligonucleotide (5.5 x 10^5 cpm/pmol) at 37 °C for 1-2 days. Cell-incorporated CRE oligonucleotides were extracted with phenol-chloroform,

precipitated with ethanol, and subjected to 20% non-denaturing polyacrylamide gel electrophoresis. UV thermal melting experiments were carried out in 200 mM NaCl, 10 mM sodium dihydrogen phosphate, pH 7.4 buffer and at the ligonucleotide concentration of 1.5 μM. Thermal denaturation profiles were recorded on a Perkin-Elmer Lambda 20 Spectrophotometer equipped with a six cell linear movement module and connected to a PTP-6 Peltier thermal controller. All data were collected and processed on a personal computer attached to the spectrophotometer using Perkin-Elmer software. Melting temperatures (T_m) were measured from first derivative plots.

EXAMPLE 13

Cellular localization of fluorescein isothiocyanate (FITC)-labeled oligonucleotide

Cells (5 x 10^5 cells/60 mm dish) were incubated with 150 nM of FITC-labeled CRE palindrome or palindromic control oligonucleotide (5'-end labeled, The Midland Certified Reagent Co.) in the presence of DOTAP in 60 mm dishes containing the growth medium at 37 °C. Six hours after incubation, the medium was removed and cells were washed 3 times with phosphate-buffered saline and were cultured in fresh growth medium. At indicated times, the intracellular distribution of FITC-labeled oligonucleotides was analyzed by fluorescence inverted microscope (Axiovert 35, Carl Zeiss).

EXAMPLE 14

Western blot analysis of CREB

Nuclear extracts were prepared by the method of Dignam *et al.* (Dignam *et al.*, Nucleic Acid Res. 11: 1475-1489) and nuclear proteins (20 μg) were separated on 12% SDS-polyacrylamide gel, and separated proteins were transferred onto nitrocellulose membrane using semidry blotting. Anti-CREB antibody (1:200 dilution, Santa Cruz Biotech.) and anti-rabbit-IgG antibody (1:2500 dilution, Amersham) conjugated with horseradish peroxidase were used as primary and secondary antibodies, respectively. Immunodetection was performed using enhanced chemiluminescence.

EXAMPLE 15

RNA preparati n and Northern blotting analysis

Total cellular RNA was prepared by the use of TRIREAGENT (Molecular Res. Center, Inc.). Northern blot analysis and hybridization of RNA with ^{32}P-labeled DNA probes were as described earlier (Nesterova *et al.*, Eur. J. Biochem. 235: 486-494 [1996]). DNA was labeled with [α-^{32}P]dCTP according to a standard protocol for random-prime labeling using Amersham multiprime DNA labeling kit. The specific radioactivity of labeled DNA equaled 3.7 x 106 cpm/µg DNA.

EXAMPLE 16

Induction of c-*fos* gene expression

Cells were treated with 150 nM CRE or control oligonucleotide for 2 days in the serum-containing medium. Cells were washed twice with phosphate-buffered saline. Cells were further incubated in the absence of oligonucleotides in the serum-free medium containing 100 ng/mL 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma) at 37 °C. At various times, cells were harvested and *fos* mRNA was measured by Northern blotting. ^{32}P-labeled probers were: 1.7 kb *v-fos* (ATCC); 4.2 kb PKC-α (kindly provided by C.A. Stein); and human β-actin (Oncor p7000 β-actin).

EXAMPLE 17

Correlation of CRE-oligonucleotides transcription factor decoy effects with cellular uptake

As shown in the Examples above, CRE-decoy oligonucleotides effectively compete with cellular *cis*-element for binding transcription factors, and interfere with the function of transactivators in intact cells. To correlate such effects of decoy oligonucleotides with their cellular uptake, ^{32}P-labeled 24-mer CRE-palindrome oligonucleotide, mismatched or nonsense sequence palindromic control oligonucleotides were incubated with MCF7 and MCF-10A cell, and cell-associated radioactivity was quantified. Within 5 hours, about 10% of the total input oligonucleotide accumulated in the cell. Incorporation continued to rise thereafter, reaching 20-25% maximum levels at 24 hours of oligonucleotide incubation as shown in Figure 11A.

In this Figure, the oligonucleotide uptake by MCF-7 cells was determined using standard procedures and as described herein. "CRE" represents CRE oligonucleotide; "CREC" represents mismatched control oligonucleotide; and "CREC(P)" represents palindromic control oligonucleotide. Data represent one of three separate experiments which gave similar results.

The amount and the rate of incorporation of the oligonucleotides were similar between MCF7 and MCF-10A cells, and between the CRE-decoy oligonucleotide and control oligonucleotides (Figure 11A). Cell-associated DNA was isolated and analyzed by non-denaturing polyacrylamide gel electrophoresis. Up to 48 hours of examination, the 24-mer CRE oligonucleotide accumulated in MCF7 cells at a size consistent with the duplex/hairpin forms as shown in Figure 11B, showing non-denaturing polyacrylamide gel electrophoresis of cell-incorporated CRE-oligonucleotide. The stability of cell-incorporated CRE oligonucleotides was determined as described above.

Consistent with these data, the 24mer CRE oligonucleotide exhibited a high melting temperature as shown in Figure 11C, showing results from thermal melting experiments conducted as described above. "8-mer" represents the 8-mer CRE; "16-mer" represents the 16-mer CRE; and "24-mer" represent the 24-mer CRE oligonucleotides. Each value is an average of two separate runs and the values are within +/- 0.5 °C.

In MCF7 breast cancer cells, within 6 hours of treatment with CRE-palindrome oligonucleotide, a strong fluorescent labeling was observed in both cytoplasm and nucleus. In addition, a large amount of labeling was also observed in the extracellular space. After 12 hours of treatment, the nuclear fluorescence becomes more intense, and extracellular fluorescence largely disappears. After 24 hours of treatment, fluorescence intensity was reduced in both nucleus and cytoplasm. This pattern of fluorescence was also observed in non-cancerous MCF-10A cells, except that the fluorescence had a more punctuated appearance. The control oligonucleotide exhibited the same pattern of fluorescence as CRE-decoy oligonucleotides.**

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described

modes for carrying out the invention which are obvious to those skilled in molecular biology or related fi lds are intended to be within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cho-Chung, Yoon S.

(ii) TITLE OF INVENTION: TRANSCRIPTION FACTOR DECOY AND TUMOR GROWTH INHIBITOR

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: MEDLEN & CARROLL, LLP
(B) STREET: 220 Montgomery Street, Suite 220
(C) CITY: San Francisco
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MacKnight, Kamrin T.
(B) REGISTRATION NUMBER: 038,230
(C) REFERENCE/DOCKET NUMBER: NIH-05032

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/705-8410
(B) TELEFAX: 415/397-8338

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGACGTCA 8

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGACGTCATG ACGTCATGAC GTCA 24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTGACGTCG GCCTGACGTC AGC 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTACGTCA 8

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGACGTCT 8

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGACGTAG 8

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGCGTCA 8

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGTGGTCATG TGGTCATGTG GTCA 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTGACCACG CCGTGTGGTC AGC 23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGCCGTCATG CCGTCATGCC GTCA 24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGACGGCATG ACGGCATGAC GGCA 24

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCTGACGTCA TGACGTCATG ACGTCA 26

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGCGTCA 7

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGGCGTAG 8

CLAIMS

What is claimed is:

1. A composition comprising one or more purified nucleic acid molecules that compete with cAMP response element (CRE) enhancer DNA for binding to one or more transcription factors.

2. The composition of Claim 1, wherein said nucleic acid molecules comprise DNA.

3. The composition of Claim 1, wherein said nucleic acid molecules comprise at least one single-stranded oligonucleotide that will hybridize to form a duplex.

4. The composition of Claim 3, wherein said single-stranded oligonucleotide comprises at least one palindromic sequence.

5. The composition of Claim 4, wherein said single-stranded oligonucleotide comprise SEQ ID NO:2.

6. The composition of Claim 1, wherein said nucleic acid molecules comprise at least one hairpin-forming single-stranded oligonucleotide.

7. The composition of Claim 6, wherein said hairpin-forming single stranded oligonucleotide comprises SEQ ID NO:3.

8. The composition of Claim 6, wherein said nucleic acid molecules comprises two hairpin-forming oligonucleotides complementary to one another in a manner wherein combining said two hairpin-forming oligonucleotides produces a cruciform structure.

9. The composition of Claim 8, wherein said two hairpin forming oligonucleotides comprise SEQ ID NO:10 and SEQ ID NO:11.

10. The composition of Claim 1, wherein said nucleic acid molecules contain modified phosphodiester bonds.

11. The composition of Claim 10, wherein said modified phosphodiester bonds are selected from the group consisting of phosphorothioate, phosphoramidite, and methylphosphonate derivatives.

12. A method for regulating gene transcription in target cells comprising:

a) providing:

i) one or more cAMP response element decoys; and

ii) one or more target cells, wherein said target cells contain cAMP response element enhancer DNA and one or more transcription factors that associate with said cAMP response element enhancer DNA; and

b) exposing said target cells to said cAMP response element decoys under conditions such that said cAMP response element decoys compete with said cAMP response element enhancer DNA for binding to said one or more transcription factors.

13. The method of Claim 12, wherein said cAMP response element decoys comprise DNA.

14. The method of Claim 12, wherein said cAMP response element decoys comprise at least one single-stranded oligonucleotide that will hybridize to form a duplex.

15. The method of Claim 14, wherein said single-stranded oligonucleotide comprises at least one palindromic sequence.

16. The method of Claim 15, wherein said single-stranded oligonucleotide comprises SEQ ID NO:2.

17. The method of Claim 12, wherein said cAMP response element decoys comprise at least one hairpin-forming single-stranded oligonucleotide.

18. The method of Claim 17, wherein said hairpin forming single-stranded oligonucle tide comprises SEQ ID NO:3.

19. The method of Claim 17, wherein said cAMP response element decoys comprise two hairpin-forming oligonucleotides complementary to one another in a manner wherein combining said two hairpin-forming oligonucleotides produces a cruciform structure.

20. The method of Claim 19, wherein said two hairpin forming oligonucleotides comprise SEQ ID NO:10 and SEQ ID NO:11.

21. The method of Claim 12, wherein said cAMP response element decoys contain modified phosphodiester bonds.

22. The method of Claim 21, wherein said modified phosphodiester bonds are selected from the group consisting of phosphorothioate, phosphoramidite, and methylphosphonate derivatives.

23. The method of Claim 12, wherein said target cells comprise cancer cells.

24. The method of Claim 12, wherein said exposing is selected from the group consisting of injection, direct exposure, oral intake, transfection, and transgenic expression.

25. A method for regulating cancer cell proliferation *in vivo* comprising:

a) providing:

i) one or more cAMP response element decoys; and

ii) one or more target cells, wherein said target cells contain cAMP response element enhancer DNA and one or more transcription factors that associate with said cAMP response element enhancer DNA; and

b) exposing said target cells to said cAMP response element decoys under conditions such that said cAMP response element decoys compete with said cAMP response element enhancer DNA for binding to said one or more transcription factors.

26. The method of Claim 25, wherein said cAMP response element decoys comprise DNA.

27. The method of Claim 25, wherein said cAMP response element decoys comprise at least one single-stranded oligonucleotide that will hybridize to form a duplex.

28. The method of Claim 27, wherein said single-stranded oligonucleotide comprises at least one palindromic sequence.

29. The method of Claim 28, wherein said single-stranded oligonucleotide comprises SEQ ID NO:2.

30. The method of Claim 25, wherein said cAMP response element decoys comprises at least one hairpin-forming single-stranded oligonucleotide.

31. The method of Claim 30, wherein said hairpin-forming single-stranded oligonucleotide comprises SEQ ID NO:3.

32. The method of Claim 30, wherein said cAMP response element decoys comprise two hairpin-forming oligonucleotides complementary to one another in a manner wherein combining said two hairpin-forming oligonucleotides produces a cruciform structure.

33. The method of Claim 32, wherein said two hairpin-forming oligonucleotides comprise SEQ ID NO:10 and SEQ ID NO:11.

34. The method of Claim 25, wherein said cAMP response element decoys contain modified phosphodiester bonds.

35. The method of Claim 34, wherein said modified phosphodiester bonds are selected from the group consisting of phosphorothioate, phosphoramidite, and methylphosphonate derivatives.

36. The method of Claim 25, wherein said exposing is selected from the group consisting of inj ction, direct exposure, oral intake, transfection, and transgenic expression.

FIGURE 1

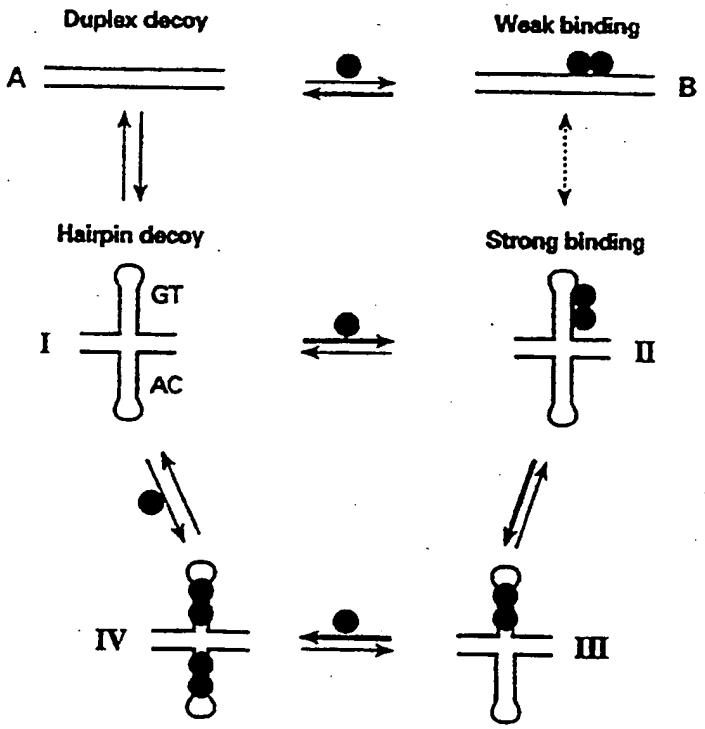

Duplex decoy
Weak binding
A
B
Hairpin decoy
Strong binding
GT
I
AC
II
IV
III

FIGURE 2

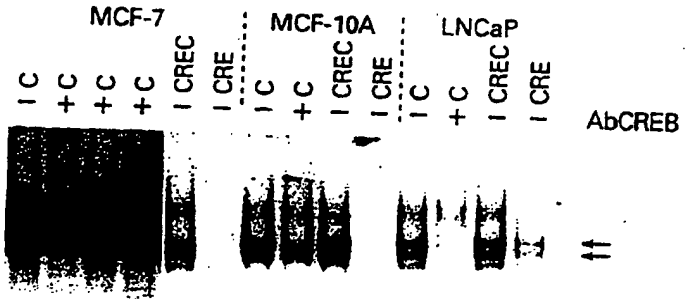

MCF-7
MCF-10A
LNCaP
- C
+ C
+ C
+ C
- CREC
- CRE
- C
+ C
- CREC
- CRE
- C
+ C
- CREC
- CRE
AbCREB

FIGURE 3

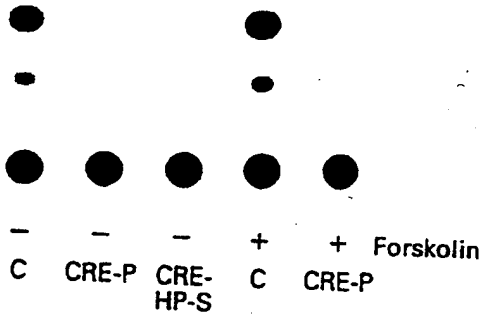


FIGURE 4

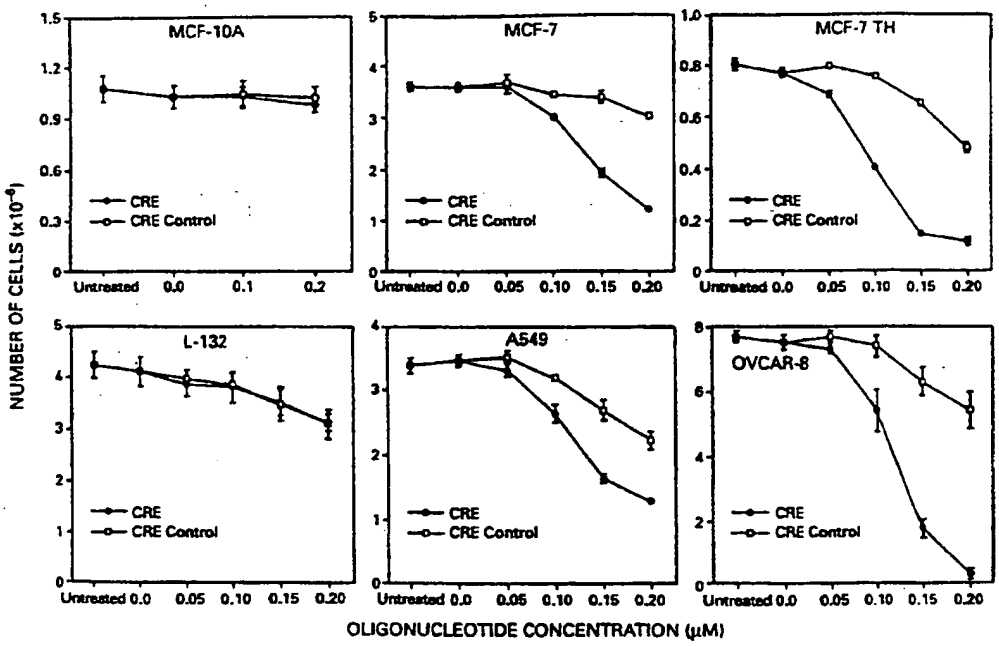

MCF-10A
MCF-7
MCF-7 TH
L-132
A549
OVCAR-8
CRE
CRE Control
NUMBER OF CELLS (x10⁻⁶)
OLIGONUCLEOTIDE CONCENTRATION (μM)
Untreated
0.0
0.05
0.1
0.10
0.15
0.2
0.20

FIGURE 5

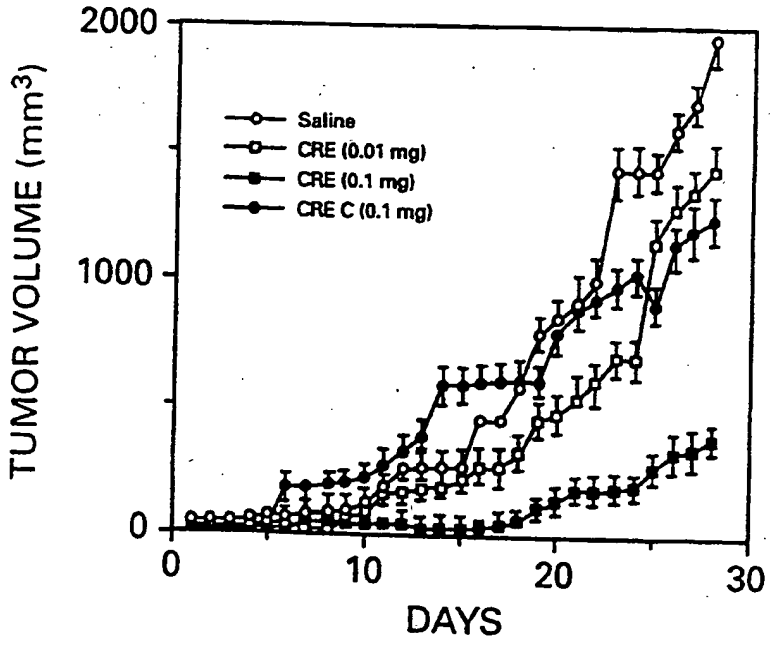

2000
1000
0
TUMOR VOLUME (mm3)
Saline
CRE (0.01 mg)
CRE (0.1 mg)
CRE C (0.1 mg)
0
10
20
30
DAYS

FIGURE 6

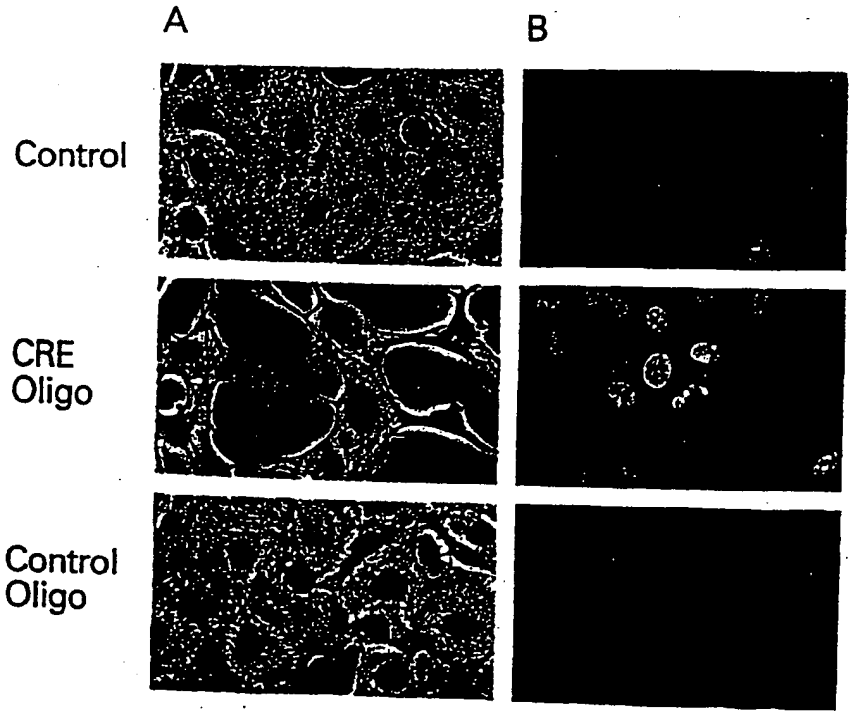


FIGURE 7

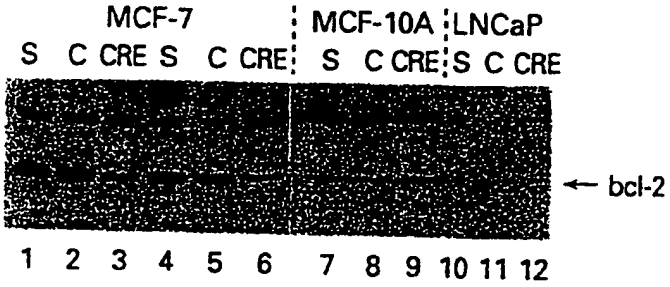

MCF-7
MCF-10A
LNCaP
S C CRE S C CRE S C CRE S C CRE
bcl-2
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 8

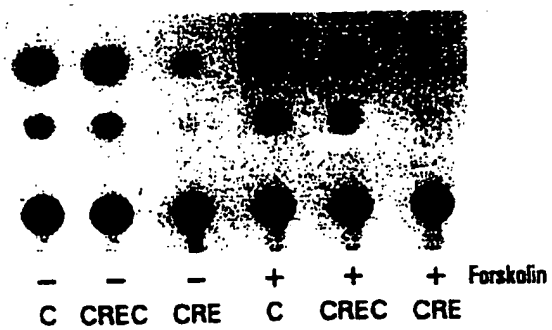


FIGURE 9

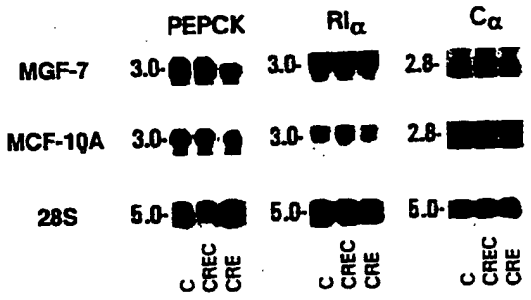

PEPCK
RIα
Cα
MGF-7
MCF-10A
28S
3.0-
3.0-
2.8-
3.0-
3.0-
2.8-
5.0-
5.0-
5.0-
C
CREC
CRE
C
CREC
CRE
C
CREC
CRE

FIGURE 10

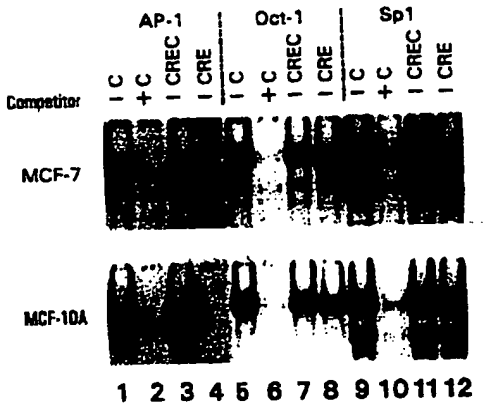
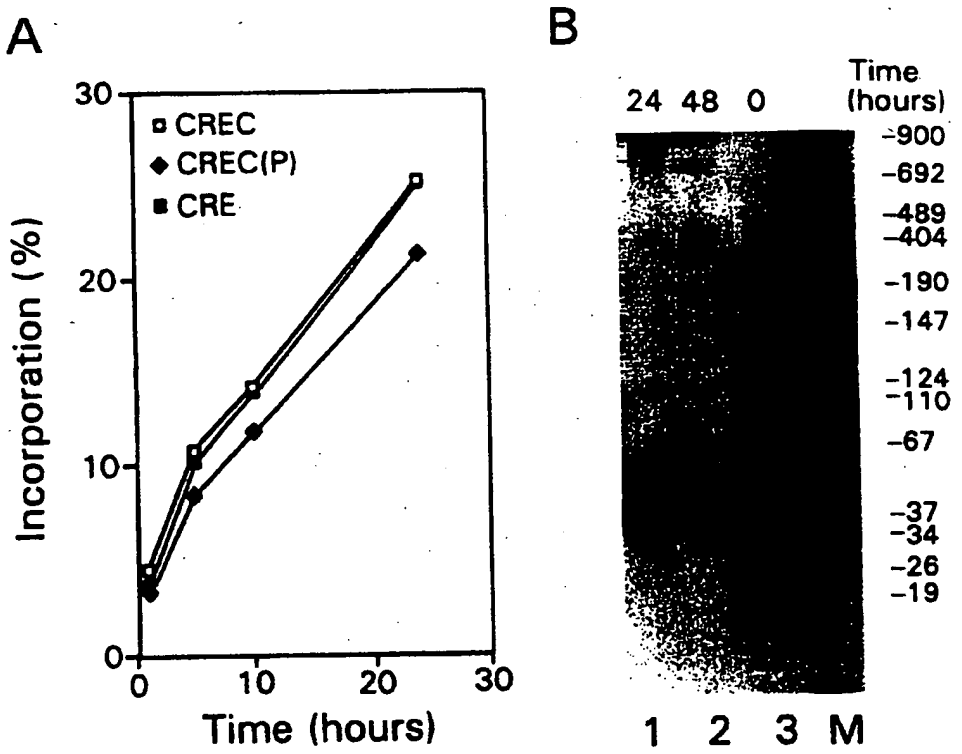

AP-1
Oct-1
Sp1
Competitor
- C
+ C
- CREC
- CRE
- C
+ C
- CREC
- CRE
- C
+ C
- CREC
- CRE
MCF-7
MCF-10A
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 11


A
B
30
20
10
0
CREC
CREC(P)
CRE
Incorporation (%)
0
10
20
30
Time (hours)
24 48 0
Time (hours)
-900
-692
-489
-404
-190
-147
-124
-110
-67
-37
-34
-26
-19
1 2 3 M

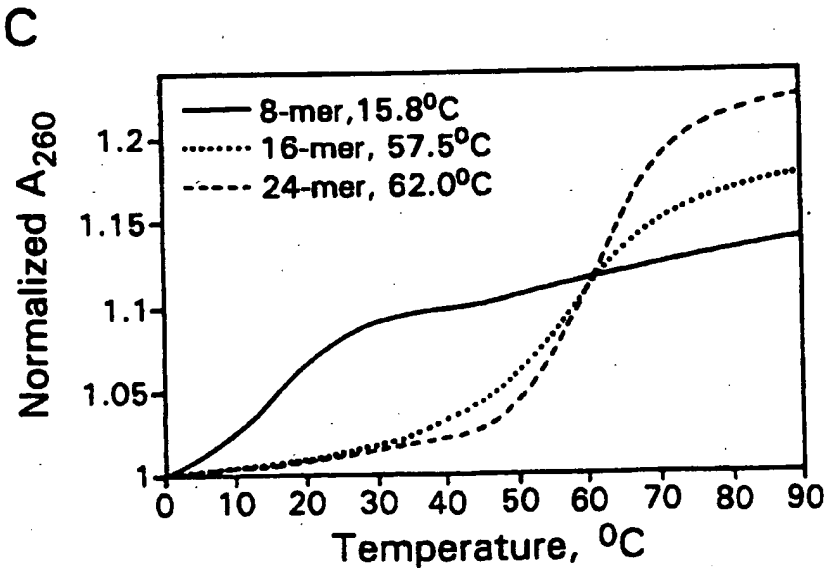
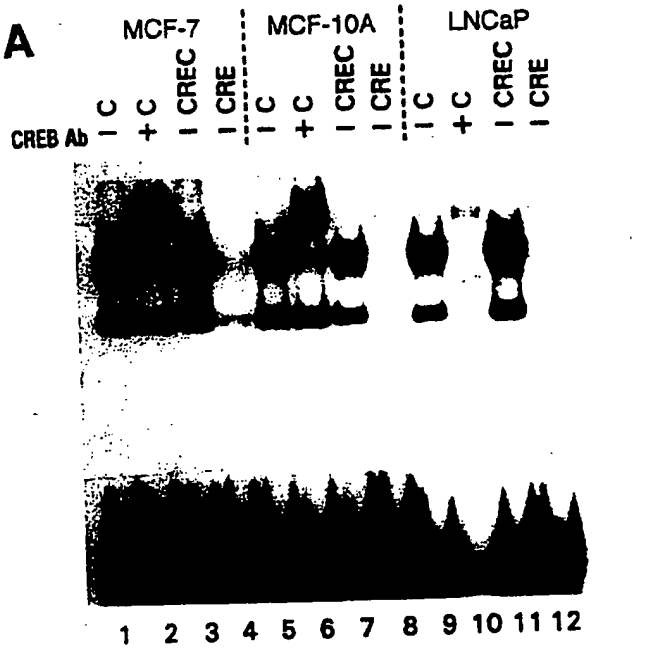

C
8-mer,15.8°C
16-mer, 57.5°C
24-mer, 62.0°C
Normalized A260
1.2
1.15
1.1
1.05
1
0 10 20 30 40 50 60 70 80 90
Temperature, °C

FIGURE 12


A
MCF-7
MCF-10A
LNCaP
CREB Ab
- C
+ C
- CREC
- CRE
- C
+ C
- CREC
- CRE
- C
+ C
- CREC
- CRE
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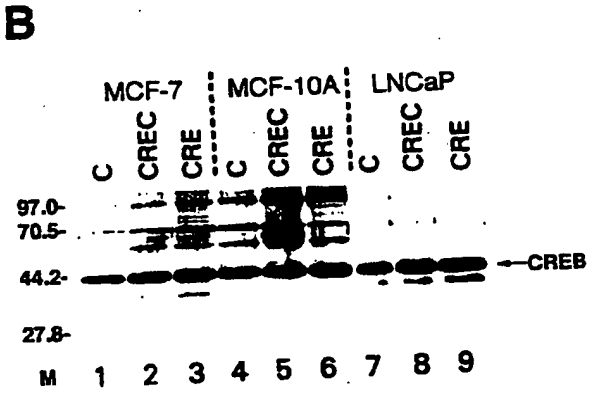
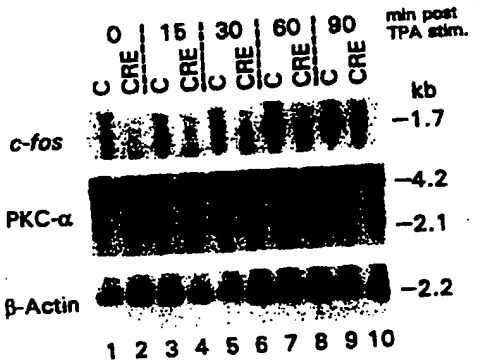

B
MCF-7
MCF-10A
LNCaP
C
CREC
CRE
C
CREC
CRE
C
CREC
CRE
97.0-
70.5-
44.2-
27.8-
CREB
M 1 2 3 4 5 6 7 8 9

FIGURE 13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15
30
60
90
min post TPA stim.
C
CRE
kb
c-fos
PKC-α
β-Actin
-1.7
-4.2
-2.1
-2.2
1 2 3 4 5 6 7 8 9 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25307

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/70; C07H 21/00; C12N 5/06
US CL : 435/375; 514/44; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 375; 514/44; 536/23.1, 24.1, 24.3, 24.31, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,683,985 A (CHU et al.) 04 November 1997, abstract, column 1, line 18-56, column 2, lines 28-34, column 4, lines 36-60, column 6, line 53-column 8, line 49, and Figure 1.	1-6, 10, 11, 12-17, 21, 22, 24 ------- 23, 25-30, 34-36
Y	US 5,641,486 A (HINRICHS et al.) 24 June 1997, column 1, lines 13-25, column 2, line 64-column 3, line 1.	23, 25-30, 34-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 MARCH 1999	Date of mailing of the international search report 09 APR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Thomas G. Larson, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25307

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Biosis, CAPlus, Genbank, Inpadoc, LifeSci, Medline, WPIDS
search terms: cre, cAMP response element, cre binding protein, cAMP RE binding protein, CRE BP, DNA binding, enhancer, decoy, oligonucleotide, palindrome, hairpin